

Can Europe ever be made to function?

The money crisis has (or should have) given the managers of the European Communities a nasty fright. The best hope now is that they will embark on a more balanced development of European institutions.

LAST week's crisis of the European Exchange Rate Mechanism (ERM) has been so thoroughly predicted that it should not have happened. It is less than a year since Britain and Italy were forced out of the system because the commercial value of their currencies differed from the notional values ascribed to them by the political managers of the European Monetary System, the governments of the 12 member countries of the European Communities (EC). People and organizations in the business of trading currencies made a lot of money last September, when the British and Italian governments stopped propping up the values of the pound and the lira, essentially by offering to pay a higher price for them than could be had on the commercial markets. When the two governments eventually caved in, it was clear even to those with no expertise in the field that the crisis would recur (see *Nature* 359, 347; 1992), but at the expense of some other government. That France and the French franc have borne the brunt of pressure in the past few weeks is an historical accident.

The fragility of the ERM is easily explained. It allows currencies to fluctuate only within a fixed percentage of a notional value defined as a weighted average of the other currencies in the system. (Until the weekend, the fixed percentage was 2.25 per cent; now it is 15 per cent.) But the commercial value of a country's currency relative to others is most directly determined by the quantity of its goods that other currencies will buy. That is why price inflation undermines the value of a currency; with the passage of time, the country's goods cost more in terms of its own currency, which then is worth less in terms of others.

Over the short run, governments can hope to fight back by increasing interest rates. The reluctance of others to buy their currencies may then be offset by the attractiveness of financial securities offering a greater rate of return than is available elsewhere. To the extent that higher interest rates are deflationary, and thus work against inflation, that stratagem may bring benefits in the longer term, even if in the short run it brings only unemployment. But, as the example of Britain last September showed all too vividly, high interest rates are not a sufficient defence against the erosion of a currency. That is what France found to its cost last week.

Irony

The irony is that all this should have happened on the heels of a long period when Europe's politicians have been pleading with their electorates (or their elected representatives)

for ratification of the Maastricht Treaty, whose chief goal is further to entrench the ERM and to replace all 12 European currencies by a single European currency, in either 1997 or 1999. The preconditions specified in the treaty (limits on inflation rates and government deficits as a proportion of gross domestic product) are unlikely to be met by either date. The British Prime Minister, Mr John Major, is probably not alone in wondering whether the high political cost of winning assent to ratification may have been too high. But they have only themselves to blame. For they are the ones who put their names to a treaty which, like many of the other instruments of European unity, is flawed not so much in detail as in principle: they put the cart before the horse.

Difficulty

Europe's money arrangements illustrate a difficulty with which too many European institutions are lumbered. The members of the EC have agreed to function as a single market in goods and some (but not all) services, and have tumbled to the discovery that the process would be more efficient if currency exchange rates were relatively stable and that it would be more efficient still if rates were fixed for all time (and thus replaced by a single new currency unit). The logic is not disputed. The difficulty is that there is more to a single currency than the name appearing on banknotes; there has also to be a central bank (legislated for by Maastricht) to act as guarantor of the single currency in external payments, and which must necessarily regulate, for example, interest rates within the EC. That in turn implies that economically less productive members of the EC will be economically further disadvantaged; manufacturers, for example, will simply move elsewhere, leaving unemployment behind them — until the unemployed depopulate the same places by looking for jobs elsewhere.

To be sure, the founders of the EC thought of that; disadvantaged regions of the EC are compensated by transfers of funds from Brussels, but only inadequately and inequitably. (Compensating Europe's farmers for the technical improvement of agricultural productivity costs much more.) In other words, the project for the monetary union of the EC, the centrepiece of Maastricht, implies that the EC will behave economically as a single sovereign unit once the treaty has finally been ratified (and the EC is renamed the "European Union"). That is the underlying inconsistency beneath the weekend's money crisis. Both monetary union and European union more generally are excellent objectives.



The fault now painfully exposed is that the project for monetary union has got too far ahead of that for general cohesion.

Will the decision that the currencies of the ERM (absent Britain and Italy) may now fluctuate in a wider range somehow save this system? The plan is that the Deutschmark and the Dutch guilder should fluctuate within the old narrow bands, but that others should be allowed to range more widely. The immediate pressure on governments such as the French will be relieved; French interest rates will fall, the government will be able to take the actions against mounting unemployment for which it has been yearning for the past several months and, paradoxical though it may seem, the franc may not depreciate very much. As the months go by, the volume of international trade within the EC should substantially increase, which will be good for everybody. In the short run, the ERM has been saved, at least in name.

The long run is another matter. The relative inflation of any one currency against another by 5 per cent a year will recreate last weekend's stresses by the end of 1995 or thereabouts, even if the wider bands are then still in place. But the optimists in Brussels at the weekend were hoping that tight bands will be restored before then. There is not much hope of that. Meanwhile, the benefits of a system in which exchange rates are so narrowly constrained that traders know in advance what they will have to pay for other people's goods has all but been lost. Last weekend's doings were a big step back from the single currency.

What should happen now? The best hope is that the past several months of turbulence will bring home to the managers of the EC the simple truth that they cannot scamper forward to monetary union faster than their other institutions will allow. A little reflection should show them that the ERM abandoned in all but name at the weekend was one with many of the attributes of a federal monetary system, but without there being a federation in any more general sense. And the truth is that Europe is not yet ready for federation.

So much is illustrated by the British government's successful protest at the use of the word "federal" in the preamble of the draft treaty negotiated at Maastricht at the end of 1991. That the EC is not now a federation, or anything like it, is more vividly illustrated by the way in which Germany was able unilaterally to arrange for its reunification at the end of 1990. It is as if, in the United States, the state of Texas, perhaps frustrated by slow progress on the North American Free Trade Agreement, had decided to reunite with Mexico, from which it was separated by force a century ago, without mentioning its plans in Washington — or anywhere else. In the German case, as it happens, consultation with fellow-members of the EC over reunification would almost certainly have won full-throated approval, and even suggestions for avoiding the mostly unpleasant consequences there have since been for the economies of the other members.

Luckily, all is not now lost, as it might have been if last Sunday's negotiations at Brussels had not preserved at least the semblance of the ERM. The best hope is that the member governments will have been sufficiently frightened that they will use the coming months to put the non-monetary institutions of the EC on a more secure and rational foundation. The

danger is that much of what needs doing will seem like a retreat from European ideals. In matters as different as environmental protection and social policy (of which the outrageously expensive Common Agricultural Policy is a part), the EC is behaving as if it were already the richest economy on the surface of the Earth, while it has merely the promise of becoming that. Europe's politicians will need courage and guile if they are to unwind, as they must, some of these extravagances without letting the vociferous army of Eurosceptics believe that they have won the day. □

Saying sorry quickly

An article on AIDS therapy published earlier this year turns out not to sustain its hopeful conclusion.

ALMOST as soon as the ink had dried on the 18 February issue of *Nature* this year, doubts were being raised about the article on page 650 of that issue by Y.-K. Chow *et al.* describing a multidrug strategy for the treatment of AIDS. The combination therapy devised was reported to eliminate HIV replication and virus breakthrough *in vitro*, the rationale being that the drug combination would force the selection of mutations lethal to the virus. The News and Views article accompanying the paper warned that the results, though important, would, like any chemotherapy, only buy time for AIDS patients. This did not prevent the results being widely trumpeted: "Graduate student cures AIDS" was the headline of a New York tabloid. But almost immediately, other researchers began to question the results. Doubts were reported first in the specialist press (*J.NIH Res.* page 30; April 1993) and more recently in the *New York Times* and elsewhere.

Now, six months after publication, Chow *et al.* have sent a retraction of part of their work to *Nature* (to be published next week). The authors admit that the discrepancies between their data and those of their critics (some of whom had earlier submitted them to *Nature*) can be explained by four previously unnoticed mutations in the reverse transcriptase of one of their HIV-1 clones. They go on to say that this development does not affect the accuracy of their other data, but it does invalidate their claim to have proved that multidrug therapy will be effective by avoiding drug resistance. It is, however, common ground between the authors and their critics that multidrug therapy may be useful for other reasons.

Whatever the outcome, the history of this article illustrates a problem now all too common in AIDS research but also in other fields. The paper by Chow *et al.* was carefully reviewed, and it was agreed that the results would be useful in planning strategies to manage AIDS. Such reports should obviously be published with speed so that beneficial knowledge may be more quickly shared. But, by the same test, retractions should also be speedy and full-throated. Publicity does not help; the scientific community is tolerant of admissions of honest error, but honest errors seem more grievous if they follow wide publicity. Sadly, there is no escape from that. □

UK faces controversy over plans to reform PhD

London. The British government is confronting a rising chorus of concern from both industrialists and academics over its plans to restructure postgraduate education in a way that would require most graduates to take a one-year MSc course before starting a three-year PhD.

The government included the requirement in its recent white paper (policy document) on science and technology, following a recommendation from the Advisory Board for the Research Councils (ABRC). Its goal is to prepare students more fully for PhD work, and to provide those who decide that one year's postgraduate study is enough with skills that employers might welcome.

The move is part of a planned overhaul of postgraduate research and training which most people agree is needed. More graduates are studying for postgraduate qualifications (see figure), and there is increasing concern that the content of taught courses and research projects does not always meet the best needs of students or employers.

The Office of Science and Technology is now discussing the compulsory MSc scheme with the research councils and hopes to introduce it in late 1995. The Science and Engineering Research Council (SERC) is being split into two new research councils.

Officials are keen to use the scheme to align support for postgraduates more tightly with each council's mission.

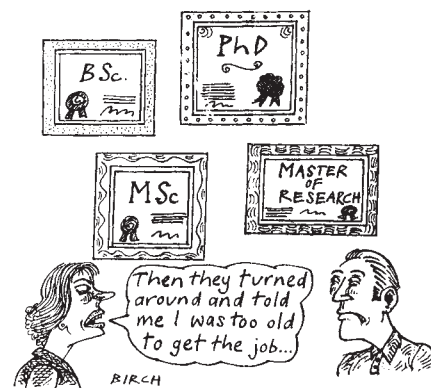
The SERC's preliminary view is that the new 40-week courses — possibly leading to a new 'Master of research' (MRes) degree — should be divided in two. One half would involve a research project, the other coursework in three areas: basic management and communication skills, research techniques specific to the discipline being studied and an introduction to advanced knowledge in that field.

Engineering employers, who are often suspicious of those holding purely academic qualifications, have in general welcomed the introduction of the new master's course. Many feel that a qualification similar to that proposed by SERC would bring Britain more in line with other countries, such as Germany and Japan.

But two of Britain's biggest employers of PhDs, the chemical and pharmaceutical industries, have come out strongly against the government's plans and have asked it to think again. The Association of the British Pharmaceutical Industry has written to Sir David Phillips, chairman of the ABRC, saying that it is quite happy with existing arrangements. It warns that moving to four-year PhD training might deter graduates from going into research because student grants are low; the Chemical Industries Association and the Royal Society of Chemistry share its concerns.

Industrialists and academics are also worried that the number of students supported by the research councils might fall by as much as a quarter as they would have to fund four years of study from the same sized pot as is now available for three. Few accept the government's response that the research councils and universities should work this out themselves. And industry has no desire to pick up more of the bill.

Professional bodies representing other academic disciplines fear that four-year PhD training would conflict with their plans for more four-year undergraduate courses. Alun Jones, chief executive of the Institute of Physics, last week wrote to William Waldegrave, the science minister, asking for a public statement "as a matter of some urgency" that those taking four-year degrees would not need to obtain



a master's qualification before beginning a PhD.

Many university scientists point out that providing taught MSc courses would put a heavy additional burden on the resources of departments: the government has said it will not provide extra money to put the new arrangements into effect. Moreover, universities are also concerned about the fate of small academic departments which lack the teaching resources for a MSc course. Students who leave to take such a course in a larger department elsewhere might choose not to return. Even those who remain would be badly placed to work as research assistants on larger projects.

Many other questions remain in the air. Would a three-year PhD still be available for the growing number of students funded from nongovernmental sources (including those from overseas)? If so, would this create a 'two-tier' PhD system? And how broad would exemptions be for those studying four-year undergraduate science courses, enthusiasm for which is growing rapidly among both students and academics?

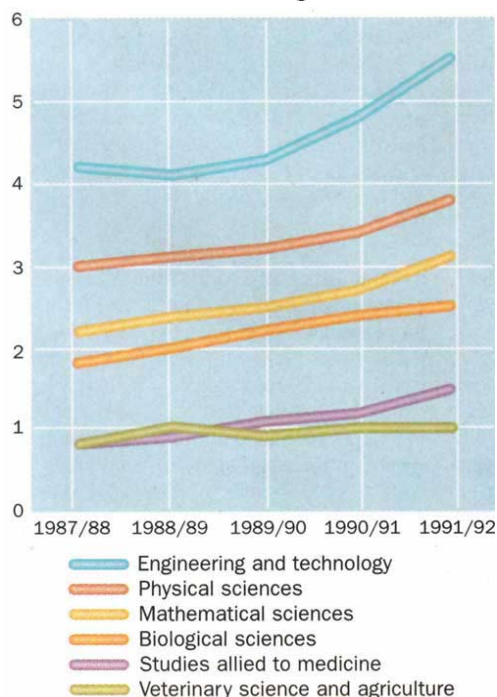
"The uncertainties are very worrying", says John Dale, professor of biology at the University of Edinburgh. He is one of many university scientists who have written to Waldegrave expressing concern. "If we are not careful, we could soon be experiencing a planning blight over future plans because there is no central guidance yet on what will be acceptable."

So far, government officials have been putting a stoical face on such concerns. They acknowledge that many complex issues will need to be resolved before the new system for postgraduates is put into place, but express confidence that solutions will eventually be found.

Meanwhile, critics of the government such as Lewis Moonie, the Labour Party's science spokesperson, say the plan epitomizes their complaint that the government has not provided adequate funding to back up the proposals in its white paper. And in the light of concerns being expressed, there are growing demands for a full-scale review of postgraduate education before the government's proposals are put into effect.

David Dickson

New entrants to postgraduate courses and research degrees in UK



US agencies urged to tighten up peer review

Washington. New guidelines have been published in Washington in the hope of improving the procedures used by the various US government agencies that make grants to scientists on the basis of peer review. In particular, the guidelines are aimed at safeguarding the confidentiality of material submitted for review, and ensuring that reviewers have no conflicts of interest.

The guidelines have been produced by a small federal agency, the Administrative Conference of the United States, which advises government departments and federal agencies on good administrative practice. They outline steps which, the conference suggests, grant-making agencies should take to help achieve both objectives.

But the proposed guidelines are not binding. And critics say the conference has drawn back from making more contentious suggestions — for example that agencies should be required to inform applicants of their legal right to complete information about why grant applications have been rejected.

The guidelines have been drawn up largely on the basis of a report by Tom McGarity, professor of law at the University of Texas. According to McGarity, peer reviewers who steal ideas have become a serious problem, and the problem is likely to get worse with the increasing financial pressure in scientific fields such as genetics. "It is one of those things on the soft underbelly of science which no-one likes to talk about," he says.

The Administrative Conference says that federal agencies should warn all peer reviewers in writing of their obligation to respect the privacy of authors, and of the penalties of transgression. As with many of the other suggested guidelines, this would

lead smaller grant-giving agencies to fall in line with existing practice at the two largest agencies, the National Science Foundation (NSF) and the National Institutes of Health (NIH).

"These two agencies have thought long and hard about it, and, compared with the smaller agencies, they have a sophisticated approach," says Charles Pou, a lawyer at the Administrative Conference.

But not everyone agrees that the big two already have it right. Jon Kalb, a geologist at the University of Texas, complains that the NSF — in contrast with NIH — fails to inform grant applicants of their full rights on the application form. Kalb won a victory over the NSF in 1987 over the reasons for which he had been denied funding for earlier research work after a protracted legal battle. His success led, in part, to the McGarity report, and to the proposed new regulations.

Lawrence Rudolph, a lawyer at the NSF, says that such information would be a waste of space on the form, since grant applicants now receive copies of the peer reviews of their applications as a matter of course. He concedes, however, that applicants do not at present automatically receive copies of the internal NSF document spelling out the full

reasons for their rejection or acceptance. Kalb says that the NSF has "gone to great lengths" not to let scientists know what information they are entitled to.

On the question of releasing information and informing applicants of their full rights, McGarity says that NIH is "the model agency, out there in front". Since Kalb's court case, he adds, NSF has "come around" in matters of substance. The main problem now lies with the smaller grant-giving agencies, including the research divisions of most government departments.

McGarity also supports the idea of putting a lay person on each peer review panel. Congress ordered the National Endowment for the Arts to do this when it found out that much art being supported by grants was subversive material inimical to the American way of life.

But the proposal has few backers in the scientific community. "Scientists were just violently against the idea," says McGarity. In its proposed guidelines, the Administrative Conference merely asks agencies "to consider panellists who are not peers but who bring perspectives relevant to the [funding] decision".

Colin Macilwain

Satellite research 'needs more money'

Washington. The US satellite communications industry faces a rout by its Japanese and European rivals because of lack of government coordination and inadequate support for research and development, claims a panel of experts.

A report compiled by leading scientists and engineers in the field and funded by the National Aeronautics and Space Administration (NASA) says the United States retains a "technology lead" in only five out of 19 key advanced technologies. In contrast, Japan leads in eight, including batteries, solar arrays and overall system design.

Europe is not considered to have an outright lead in any of the technological areas considered. But the report still applauds what it sees as the successful industrial policy led by the European Space Agency (ESA) in satellite communications.

"NASA just isn't supporting the industry to the extent that ESA and NASDA [the Japanese space agency] are", says Burton Edelson, a former senior administrator at NASA who acted as a co-chairman of the 12-strong panel. Its report was based on visits by the panel to Western Europe, Japan and Russia, and was released last week by the International Technology Research Institute at Loyola College, Maryland.

According to Edelson, almost all of NASA's heavy spending in the past has

been directed to distinct programmes. As a result, he says, no money has been spent on important generic technologies needed to ensure the future strength of the United States in what is becoming a fiercely competitive field.

The report does not explain why corporations that have previously benefited from NASA's largesse now find they are unable to crush upstart competition from Europe and Japan. But Edelson denies that this reflects badly on the way in which NASA spends its \$15-billion annual budget. "It's more a reflection of the work NASA hasn't done", he says.

"We are arguing that NASA should spend a higher percentage of its money on satellite communications, not that it should spend more money overall," says Edelson. He points to the \$200 million that NASA spends each year on microgravity research as an example of money that could be better spent in an area such as satellite communications, where commercial spin-off is assured.

The publication of the panel's report coincides with concern being expressed by the satellite communications industry that NASA's Advanced Communications Technology Satellite programme, whose budget peaked at \$100 million, is now nearing its end, but that no replacement is in prospect.

Colin Macilwain

McBride struck off

Sydney. William McBride, who first alerted the world to the dangers of thalidomide, was struck off his state medical register in Australia last week because he falsified scientific data.

The Medical Tribunal of New South Wales, sitting in Sydney, voted 3 to 1 on the decision, which effectively ended the 67-year-old researcher's career, but expressed its "profound regret" over the move.

The tribunal found that McBride's character was "flawed", following an earlier finding that the researcher had altered and falsified scientific data.

After a marathon hearing in February, the tribunal found that McBride had changed data on the effect of a drug, related to the morning sickness drug Debendox, on a group of rabbits, to support his views that the related drug caused birth defects.

Mark Lawson

Irish geneticist takes over the helm at EMBO

Munich. Frank Gannon, professor of molecular genetics at University College Galway (UCG) in Ireland, is to become head of the European Molecular Biology Organization (EMBO).

One of Gannon's first tasks when he begins in January will be to renegotiate



Frank Gannon

funding from the European Molecular Biology Conference (EMBC): their agreement must be renewed by spring 1995. The 16 member states of the EMBC this year gave EMBO DM16 million (US \$10 million) to run its programmes,

courses, workshops and fellowships.

These negotiations will probably touch on the sensitive subject of EMBO's relations with the European Commission. The commission's Human Capital and Mobility fellowship is open to scientists from all disciplines and overlaps with EMBO schemes. EMBO officials complain that because the commission's grants do not always conform to local pay scales (see *Nature* 361, 196; 1993), researchers may find EMBO fellowships less financially attractive. Gannon's style will be to cool things down: "We will be looking for ways of working together to ensure the best interests of Europe's young molecular biologists by seeking out cooperation and partnership."

Gannon, who is 45, was an EMBO fellow himself, in Pierre Chambon's laboratory in Strasbourg. After six years there he returned to Ireland "to establish a serious effort in molecular biology". He now leads a team of 15 researchers at UCG.

He also heads Ireland's National Diagnostics Centre. This is part of BioResearch Ireland, a government-sponsored contract research organization. Gannon began to build the centre from scratch five years ago. It now has 45 employees and is expected to have a turnover of at least IR£0.5 million (about US\$1 million) this year.

Gannon will not give up research completely: he will continue to work with both his research group and the centre for two to three years. He might then transfer his research to Heidelberg.

In the first year, Gannon will share his new job with his predecessor, John Tooze, who now works at the Imperial Cancer Research Fund in London. Tooze will also continue as editor of the *EMBO Journal*, whose office will stay in Heidelberg.

Alison Abbott

Policy goals 'colour choice' of centres of excellence

Tokyo. Japan's Science and Technology Agency (STA) is expected to announce late next week the selection of three "centres of excellence" from the country's many government-funded research institutes. Each will receive a welcome boost in research funds, under a new programme introduced by the STA to stimulate the work of national laboratories. But some of the criteria by which the centres are being selected seem to have little to do with academic excellence.

The idea of creating centres of excellence — or COEs, as they are called in Japanese — is the latest manifestation of the government's desire to build up national research institutions with the same status and prestige as organizations such as the Massachusetts Institute of Technology in the United States, or Germany's Max Planck institutes.

Research institutes financed by a wide variety of ministries and agencies have applied for funding from the STA scheme, which will provide ¥400 million (\$3.8 million) a year for each selected institute over the next five years. Curiously, however, some of Japan's best institutes have already been turned down, and only about six remain in the competition.

The Institute of Physical and Chemical Research (RIKEN), for example, widely acknowledged to be the best and most international of the institutes funded by the STA, was advised not to apply. Preparations to do so had already been made by the institute. But agency officials told RIKEN that in the first year they want to give the money to laboratories that are totally funded by the government, and RIKEN has some small independent sources of funds.

Another surprise has been the elimination of the National Cancer Center Research Institute in Tokyo. This operates under the Ministry of Health and Welfare, and also enjoys a strong international reputation. But the institute was not put forward by the ministry for the extra funding because it is expected to receive a substantial amount of additional money through an extension of the ten-year cancer research programme that ends next March (*Nature* 361, 672; 1993).

The ministry's National Institute of Health in Tokyo, which proposed to do research on AIDS, was also eliminated in the early rounds. Instead, the ministry has chosen to put forward the less well known National Cardiovascular Center Research Institute in Osaka.

The Electrotechnical Laboratory, one of the best known institutes under the Ministry of Trade and Industry (MITI), has also been eliminated from the selection process. So, too, has MITI's new National

Institute of Material and Chemical Research in Tsukuba. Rather than put either of these forward, MITI has chosen to back the National Institute of Bioscience and Human Technology in Tsukuba, which is proposing a broad programme of research on signal transduction in humans, to be carried out by molecular biologists, protein chemists and cell biologists.

STA officials refuse to discuss details of the selection procedure until the three winners are announced next week. But, according to the head of one institute, government officials decided not to include any national institutes from Tokyo in order to allow money to be pumped into local regions as part of a general government policy of decentralization.

Others suggest that each science-related ministry and agency has decided to back a single candidate — regardless of the number and quality of their research institutes — in order to maximize its chances of success. Japanese officials still seem reluctant to accept that true academic excellence will be achieved only when a system of free and open competition, regardless of government affiliation, is introduced. **David Swinbanks**

Is pressure off Japan to contribute to SSC?

Tokyo. Japan is asking itself whether the United States is no longer pursuing it for a contribution to Superconducting Super Collider (SSC). The reason is that US president Bill Clinton did not raise the issue during meetings with Japanese prime minister Kiichi Miyazawa either in Washington last April or in Tokyo last month.

The simple explanation is that the two leaders were too busy discussing trade and economic matters in the limited amount of time available for the meetings (one and a half hours in the case of last month's G7 meeting in Tokyo). But the difference between Clinton and George Bush, who always brought up the SSC in meetings with his Japanese counterpart, has not gone unnoticed in Japan.

The United States and Japan last held formal discussions on the matter a year ago through the joint US-Japan SSC working group. Its meetings were suspended when Clinton was elected president, and the United States has made no move to reconvene them. Resumption seems unlikely before the crucial vote in the US Senate in September, following defeat of budget appropriations for the SSC in the House of Representatives last month.

David Swinbanks

British voters force rethink of global warming strategy

London. The voters of Christchurch in Hampshire gave Britain's Conservative government a sharp warning last week that fiscal instruments applied in isolation from broader social policies are unlikely to provide an acceptable strategy for combating global warming.

A key factor in the government's worst by-election defeat since 1945 was its decision in March to introduce value added tax (VAT) on domestic heating fuel. The Treasury justified this move on the grounds that this was needed if the country was to reduce carbon dioxide emissions. But Christchurch voters were more concerned about its impact on low-income groups, particularly old-age pensioners.

Ironically, the defeat came three days after the Minister for the Environment, John Gummer, announced further measures to the same end. The government, he claimed, could now meet the commitment it made at last year's earth summit in Rio de Janeiro: to reduce carbon dioxide emissions to their 1990 levels by the year 2000.

To meet this, Britain will have to cut such emissions by 10 million tonnes of carbon, 6 per cent of what was previously estimated. The government had planned to achieve 15 per cent of this reduction by imposing VAT on heating fuel, and a further 15 per cent by increasing taxes on petrol; these goals will have to be reconsidered following its decision to halve the planned VAT rate on heating fuel (and possibly to abandon it all together in the light of last week's defeat).

The government's main strategy so far for curbing carbon emissions has been to seek measures that "go with the grain of the market," instead of resorting to direct regulation. Measures previously announced, for example, have focused on informing individuals and businesses about ways to save energy and giving them financial incentives to do so, for example through the creation of an Energy Saving Trust.

Last week's package continued in the same vein. The main quantified target was a goal of reducing energy consumption by 20 per cent of 1990 levels that is being set to all government departments. Gummer also announced he would help the government's Energy Efficiency Office in its task of advising small businesses by creating Local Energy Advice Centres.

Many of those who support strong action to curb global warming have welcomed the new measures. But they warn that they do not go far enough in important areas, such as curbing vehicle emissions. "We felt that the government could, for example, have adjusted tax on company cars to give an incentive for greater energy efficiency," says Chris

Hampson, head of the global warming working group of the government's Advisory Committee on Business and the Environment.

Others claim that the results of the Christchurch by-election show a growing awareness that fiscal instruments alone (such as the blanket imposition of VAT on heating fuel) are not only unfair (those affected most are low-income households in which heating bills account for more of the budget) but are also not very effective. "The problem is the inelasticity in demand," says Ben Plowden, energy campaigner of the Council for the Protection of Rural England. "Those who cannot afford to improve the efficiency with which they use energy just end up paying more, not consuming less. We need an approach that combines price signals and the ability to respond to them."

Environmentalists are now joining social action groups to launch a campaign to persuade the government to adopt just such an approach. They welcome government promises to reimburse low-income families for the extra costs of heating fuel. But they also demand that it invest heavily to increase the energy efficiency of low-income households.

The political fall-out from Christchurch is expected to make the government more responsive to such arguments. So too is its wish to show other members of the European Communities that it is more desirable to reduce carbon dioxide levels by introducing carefully designed (and politically acceptable) packages of incentives in each state than to impose a blanket carbon tax.

David Dickson

Constitution torpedoes French university reform

Paris. A bill that would have given French universities a greater say in their own affairs was scrapped last week because it conflicted with the constitution. Parliament had adopted it by a majority early last month.

The bill would have let universities opt out of the 1984 Savary law. This specifies national norms for such things as the appointment and role of a university council, and the statutes and financial and organizational structure of a university.

The constitutional council ruled that the bill was illegitimate because it would have let universities fix their rules: article 34 of the constitution says only parliament can do this. It also ruled that the bill threatened the constitutional liberty and independence of university teachers by letting outsiders onto university councils.

François Fillon, minister for higher education and research, last week expressed his "regret" at the decision. He must have been surprised too. The bill was a clever alternative to attempting to replace the Savary law completely and to impose autonomy on the universities: it was meticulously engineered to ensure that reforms gained momentum gradually while avoiding dissent among university teachers and student opposition on the streets (see *Nature* 364, 5; 1993).

The government's advisers had assured it that the bill did not conflict with the constitution. The only constitutional route to giving the universities greater autonomy now open to Fillon is to remove the Savary law from the legislature. And last week he announced his intention to do so after the parliamentary elections in 1995: as "the only way to modernize French universities".

Declan Butler

UK health authority suspends Allain

London. Jean-Pierre Allain, the former head of research at the French National Centre for Blood Transfusion, was last week suspended as director of East Anglia regional blood transfusion services following his imprisonment by a French court over his role in allowing haemophiliacs to be treated with blood infected with HIV (*Nature* 364, 269).

Allain held a joint appointment as professor of transfusion medicine at the University of Cambridge and director of the regional blood transfusion service. Earlier this year, the health authority set up an ethics committee chaired by the philosopher Baroness Warnock. This concluded Allain had acted in a way "consistent with medical ethics" and considered that he was fit to continue in his position.

However the regional health authority now says it would be "inappropriate" to continue to pay Allain as he is "unavailable to conduct his duties". The University of Cambridge says it has granted Allain leave of absence. Both the university and the health authority say they will reconsider their position in the autumn, after the French Supreme Court decides whether to grant Allain a further appeal.

Since Allain's imprisonment, his British colleagues have spoken out against the verdict. In a letter to the *Lancet* (24 July), 37 staff from the department of haematology at the University of Cambridge describe the verdict on Allain as 'bizarre' and promise that they will "work to right this injustice."

Declan Butler

Italian universities 'must be more efficient'

Munich. Italy's parliament has welcomed proposals by the new science minister, Umberto Colombo, to reform the country's disorderly universities. Although time is not on Colombo's side — the interim government will be replaced in a general election next spring or even sooner — his forthcoming three-year plan may well set the agenda for the incoming government.

Italy's universities face several major problems. Although they produce a few excellent scientists, says Colombo, the general standard is low. Costs in many universities have soared unreasonably; cost effectiveness can vary twelvefold between campuses. Many universities are also seriously overcrowded, and the average drop-out rate (64 per cent) is almost double that of other large European countries.

"We need to apply a magnetic field to the disorderly world of university research," says Colombo, whose full title is minister for universities and research. He is not the first to think so: successive governments have attempted to reform the university system with little success. Italians are now impatient for change, not just prepared to tolerate it; and for the first time real reform seems possible. Nevertheless, Colombo is proceeding carefully: "If you push too hard against resistance, in a few years everything will return to how it is now."

One of Colombo's reforms would be to make universities more responsible for their spending. He would let them manage their entire budget, and provide funding as a lump sum instead of on an item-by-item basis. He intends this to stimulate greater

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UNAVAILABLE
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REASONS

cost effectiveness. It would also mean that universities that overspend could be penalized. "We do not intend to continue funding inefficiency," says Colombo.

If universities have to be seen to be acting efficiently, he says, the problem of the much criticized system of appointing professors, which is alleged to lead to fixing, might also disappear.

He also wants to slash the generous subsidies universities enjoy: "we can no longer afford them". Such subsidies also contribute to the problems of overcrowding and high drop-out rates: many students go to university because they will be better off than if unemployed — fees are cheap and they benefit from highly subsidized facilities such as canteens. Young people sometimes use universities "like parking lots", says Colombo, using an analogy well worn by exasperated academics.

The major problem facing university research is unfair distribution of public funding. The state provides money for fundamental (60 per cent) and applied research

Umberto Colombo followed Luigi Berlinguer into the ministry for universities and research in May. Berlinguer, rector of Siena University, held office for only a few hours before his PDS party (ex-communists) resigned from government. The interim government appointed Colombo, a physical chemist with no political allegiance, as an impartial expert. Until recently, he was chairman of the Italian National Agency for New Technology, Energy and the Environment. He is president of the European Science Foundation.

(40 per cent) either directly or via national research councils. But the National Universities Council's 12 discipline-orientated review committees lack a formal referee system and appropriate *post hoc* evaluation. They try to keep everybody happy by giving each a little money.

Bad enough for basic research, but a disaster for applied research, says Colombo, who wants to introduce conventional refereeing and formal evaluation. Money for applied research should be divided with clearly defined priorities in mind, he believes. "You can't tackle big problems such as climate research with small grants to individuals."

The National Institute of Nuclear Physics, which deals with subnuclear and high-energy physics, has always done very well. Its success proves some things can work well in Italy, says Colombo. But it will not be so generously funded in future; more money must be made available for other research areas of more direct relevance to the Italian economy which have not received as much attention.

Colombo also wants more collaboration between universities and industry. It can work. In the 1950s, scientists at the Polytechnic of Milan (led by Giulio Natta, the 1963 Nobel prizewinner in chemistry) and the Montecatini company developed polypropylene in Italy; more than one-hundred of the company's staff worked for years as researchers at the polytechnic.

But this unfortunately is an isolated success story. Corruption has shattered large industry, and the hundreds of thousands of small companies in Italy have little interest in research: they prefer to redesign products developed elsewhere.

"We hope the new wave of morality and rebellion in the country will encourage the universities to act," says Colombo. But the universities are constitutionally independent, and he realizes that besides making them financially responsible he can only cajole them into change. Colombo lives each day as if he were minister for life, he says, while ready to pack his bags at any moment. That's life in Italy now.

Alison Abbott

Alison Abbott

Time runs out in space research dispute

Munich. A deadlock between the board and scientific committee of the Italian space agency (ASI), which has resulted in no research funds being given for almost two years, will die a natural death next week when the board is disbanded at the end of its five-year term.

Ultimate responsibility for the mess lies with the former government which failed to take decisive action to restore order, says Umberto Colombo, research minister in Italy's interim government. But ASI's board and scientific committee were also at fault, he says.

One of the main reasons for the deadlock was an argument over what proportion of the budget should be spent on national space programmes and what on contributions to the programmes of the European Space Agency (ESA). Escalating internal feuds ended up in court, but researchers have still received no funding.

The board, says Colombo, should have

consulted the scientific committee on decisions involving ESA: the committee argued that ASI's commitments to ESA were a drain on national programmes. The scientific committee should also have been more selective in giving out grants, he says: they wanted to fragment funding too much.

But the main problem was simply one of bad management. By law, the chairperson and director of ASI must be expert scientists. Colombo believes expertise in strategic management would be a more helpful qualification if ASI is to avoid similar mistakes in future.

A new board will be a critical opportunity for change, says Colombo. Italy, he says, will be more selective about space research in future. ASI's unrealistic promises overstretched budgets. Italy will now support the trend in Europe towards a deceleration of the space programme combined with greater international collaboration.

CHINA IN BRIEF

Beijing. The Chinese Academy of Sciences (CAS) has broken a long custom of making closed appointments of senior bureaucrats by announcing that one of its institutes — the Institute of Geophysics — will publicly advertise for a new director.

A CAS spokesman, Gua Chuan-Jie, called the move “a new attempt to deepen reforms currently being undertaken within the academy”. The academy has made reform of rigid personnel policies a priority, and is likely to open other jobs to external candidates.

Geophysics professors under 55 years of age may apply for the job. The successful applicant will get the same privileges as government officials of equivalent rank. The spokesman says: “We do not want a bookworm. The new director should be capable of dealing with society.” Applications close on 8 August.

■ The administration will test a new and powerful satellite launch rocket, Long March 3a, within the next three months. It plans to use it to launch a domestic telecommunications satellite early next year. A larger thrust version — Long March 3b — is in the pipeline. It should be ready to launch foreign satellites within two years.

China has already launched five foreign satellites, for Sweden, Pakistan and Australia, and has contracts to launch another three. But although China can offer competitive fees, its existing Long March 2 rocket can carry only a small payload. Long March 3a will be able to carry 2.5 tonnes and version 3b 4.8.

But loss of an Aussat-2 satellite last year on its launch by a Long March 2 rocket has cast a shadow over the programme. Scientists and engineers working on the new launchers have made improving reliability a top priority.

■ China is to create a national network of nature reserves, as part of an effort to cooperate with United Nations programmes and to protect the Chinese countryside from burgeoning development.

China now has 708 nature reserves covering 6 per cent of the land. But most have inadequate infrastructure, lack information services and are poorly managed. Conservationists are frustrated by the lack of coordination between reserves, which fall under various central and provincial government departments.

The new programme was announced by Zhao Xian-Ying, secretary general of the national committee on the UNESCO-led Man and the Biosphere (MAB) programme. It will try to coordinate management of existing reserves, including nine chosen to take part in the international MAB programme.

China plans to have 1,000 nature reserves by the year 2000. But critics say that the country still lacks a coherent conservation research programme to accompany the proposed network.

Yao Qin Li

Election focuses concern on future of science in S. Africa

Cape Town. South Africa is studying ways to increase graduate numbers in science, medicine and engineering, and maintain its research base, in the run-up to next year's democratic general election.

Both the ruling National Party and the African National Congress (ANC) agree that the education system must produce more technologically competent students. The problem is that the large expansion of the universities over the past decade has mainly benefited graduate numbers in arts and social sciences: the percentage of science and engineering graduates has fallen from 17 to 13 per cent, and those in medicine from 11 to 7 per cent.

This is mainly because most Africans go to schools that lack mathematics and science teachers, and consequently cannot meet the entrance requirements for scientific university courses. Even those who have studied science frequently lack laboratory experience and have poor English skills.

One possible solution is the so-called bridging programmes. These give disadvantaged students training in communication skills and hands-on laboratory experience. The largest of these is the “College of Science” programme started at the University of the Witwatersrand in 1991.

There students complete a special two-year curriculum after which they can enter the second year of the degree programme. It has since almost doubled its intake of science students. Recently it also extended the programme to engineering.

“This system could serve as a model for colleges in a new tertiary education system,” says Robert Charlton, the vice chancellor. One problem is that these students do not qualify for state subsidies. The programme and smaller ones at the University of Cape Town in medicine and engineering have had to rely on funding from the private sector. But the new government is likely to make such programmes a priority for funding.

The ANC has also speculated about adopting the British model of setting national quotas of subsidized places for each discipline and allocating them among the universities. “I think it is a great idea, but I doubt the new government will have the political will to implement it,” says Charlton. Its political constituency would probably object to introducing such a system until imbalances in primary and tertiary sectors have been redressed.

One thing is clear: expansion of postsecondary education is unlikely to be oriented towards the universities. Enrolment in higher education (17 per cent of those aged between 20 and 24) is already skewed towards the universities (62 per

cent). Future growth will probably be directed towards technikons (23 per cent) and education colleges (15 per cent).

The other main problem facing South African research is that the general standard of its universities is low. They are also heavily oriented towards producing graduates in the arts and social sciences. Many feel that some universities should be downgraded to colleges offering only undergraduate degrees, to concentrate scarce research resources on a few “research” universities. “However sensible this may be, it will be politically very difficult to accomplish,” says Charlton.

Khotso Mokhele, vice-president of the Foundation for Research Development, the state funding agency for science, is not against the idea if some research universities would be historically black institutions. “The University of Fort Hare, for example, is an important symbol for black people all over the continent,” he says.

Stuart Saunders, vice chancellor of the University of Cape Town, is optimistic about the future of both the Universities of Witwatersrand and Cape Town. They have a greater combined research output (in terms of publications in the Institute for Scientific Information's *Science Citation Index*) than the eight next-ranking universities in South Africa.

He has also suggested that a Californian model — where different post-secondary institutions offer maximum accreditation with each other to simplify transfers between them — might be preferable to creating distinct research and teaching universities. Saunders also thinks the government should direct research funding away from research councils, state laboratories and strategic industries to the universities, which can use it more efficiently.

“I think that the new government will realize that the retention of this research base is essential not only for the development of this country, but for the whole of sub-Saharan Africa,” he says. Charlton, is more circumspect: “Resources are going to decline, and we need therefore to allocate them more selectively. The difficulty will be to persuade everybody that selective allocation of resources is the only way to go.”

Whatever the outcome, the universities will next year face the prospect not only of answering to a democratically elected government but also the possibility of being funded through regional authorities instead of the Department of National Education. This will depend on the outcome of continuing constitutional negotiations about what functions the government should delegate to the regions.

Michael Cherry

Belfast university reverses record on discrimination

Belfast. Three years ago, the reputation of Northern Ireland's oldest university was seriously damaged by charges of discrimination against the Roman Catholic community. The charges were given additional weight by an independent survey published in February. But figures announced last month show that by tightening up of employment practices, Queen's University, Belfast, has achieved a 50 per cent rise in the proportion of Catholics among staff born and educated in Northern Ireland.

The university was established in 1845 under an act that specifically prohibited religious discrimination. It was also one of the first employers in Northern Ireland to announce its compliance with fair employment legislation passed in 1976. The local community was therefore particularly shocked by the findings of a three-year investigation by the Fair Employment Agency, published in 1990, which revealed clear evidence of religious discrimination.

Using data from 1987, the agency reported that only a quarter of locally recruited staff came from the Catholic population, which at that time made up 36 per cent of the inhabitants of the local catchment area. The medical faculty was the worst offender; only five per cent of its locally recruited staff were Catholics.

The same trends emerged when the recruitment policies were placed in a broader context. More than half the academic staff are recruited from outside the province; but of those coming from Northern Ireland, 80 per cent of the nonclinical staff (and 95 per cent of the clinical staff) were Protestant.

The publication of the report was followed by a string of tribunal cases brought by individuals claiming religious discrimination. Two of these, settled out of court in June last year for £40,000, precipitated wide student unrest, and led to a demand from the Catholic population for the resignation of Gordon Beveridge, the vice-chancellor.

In response, Beveridge commissioned an independent investigation into employment practices at the university. The results, published in February this year, confirmed the Fair Employment Agency's conclusions, and made specific recommendations for improvement. It also suggested that the university would have found it difficult to defend the controversial discrimination cases if they had come to trial.

Beveridge immediately accepted the 93 recommendations that arose from the investigation, and acknowledged responsibility for discrimination in the past — moves which helped to head off a further wave of bad publicity. He pointed out that some of



Queen's University, Belfast

the recommendations were already being put into effect; indeed, figures published by the university last month show that the proportion of total staff born and educated in Northern Ireland has risen from 19 to 29 per cent in the past six years.

Beveridge also set up an equal opportunities committee, made up of six non-university members of senate, to oversee the implementation of the recommendations. The changes will be introduced over a period of two years according to a detailed timetable that includes elaborate recruitment procedures and frequent analyses of staff numbers.

The main reason for the situation getting out of hand, according to the investigation, had been a lack of personnel policy. The staff of academic departments wrote their own advertisements, and placed them only in the Protestant press. Since April, all vacancies at the university have been dealt with by the personnel department, and have been advertised in three local newspapers.

Positive discrimination is illegal. But the university is already committed to a greater degree of affirmative action than any other university in the United Kingdom. From June, vacancies in areas where Catholic participation is particularly low have been advertised with a rider specifically encouraging Catholics to apply. And departments have been asked explicitly to consider their own religious bias when choosing between applicants who are otherwise equally qualified.

The prime black spot remains medicine. But close on its tail are the library, and the cleaning and administration departments. Many are unhappy that these departments are not taking seriously enough the requirement for change. Indeed, there has been no significant change in staff ratios in the medical school and libraries.

University officials say that the ability to alter staff ratios is directly related to the rate of staff turnover. But Tim Atwood, director of Belfast's Citizen's Advice Bureau and a member of the university senate, is sceptical. The medical school and library should be called to account and made to justify their poor showing, he says.

Alison Abbott

Russia to identify key centres

Moscow. After long procrastination, the Russian government has finally begun to identify research institutes which are to be known as state scientific centres, and which will benefit from increased budgets and other privileges.

In a decree that has now been issued, President Boris Yeltsin has nominated 33 institutes as state scientific centres (SSC). Although institutes of the Russian Academy of Sciences (RAS) make up a large proportion of those (almost 300) seeking this new status, none has yet been chosen.

To be nominated, a research institute must be considered to perform research of great value to the state. Most of the first 33 are applied research institutes such as the Physical Power Institute (Obninsk), the Central State Institute of Aviation (Zhukovskii), the State Optical Institute (St Petersburg) and the Institute of Applied Chemistry.

The new centres are being financed by a special allocation of 57 billion rubles, which translates into a 30–40 per cent increase of the budgets of the chosen centres, and thus of the salaries of their researchers. Although

the new status of SSC has only now been decreed, the Ministry of Science has apparently been paying the extra budgets on its own responsibility since the beginning of the year.

Many directors at the SSCs say that they are now less worried about the survival of their institutes than by the problems caused by the deprivation of the scientific schools, the need to replace obsolete equipment and the freezing of posts at their institutes. Even the comparatively small increase of salaries has apparently been enough to stem the outflow of people, and occasionally to attract newcomers.

The omission of RAS institutes from the first list of SSCs owes less to an objective judgement of their quality than to the resistance of the RAS leadership to the proposal that some institutes should be given special status. But ministry sources expect that the RAS will eventually concede that some of its institutes should be nominated as SSCs, if only because all research institutes must now have more than one source of funds if they are to survive.

Vladimir Pokrovsky

Allain's warnings unheeded

SIR — I write to respond to your leading article (*Nature* 364, 267; 1993) on the case of Jean-Pierre Allain. The trial and the verdict do indeed raise important issues of principle, but they go much further than the issue of when "whistle-blowers should blow the whistle". More important, as the Royal College of Pathologists argued in its statement earlier this year, is the issue of whether "doctors who give their professional opinions in good faith . . . can be held legally responsible for the failure of that advice to be taken".

You appear to blame Allain for "not blowing the whistle between the beginning of 1984 and October 1985". The *Lancet* points out "Between knowledge and action there is always an interval because the knowledge itself may prove insecure and because it takes time to move from research base to action" (342, 188; 1993). It is therefore not just a matter of detail that your starting date "beginning of 1984" is almost exactly one year too early.

What did Allain (or anybody else) know early in 1985? In 1983–84, Allain coordinated a study of the immunological and virological status and type and origin of blood products in French multi-transfused patients. The data were analysed by December 1984, first submitted for publication on 7 January 1985, eventually accepted for publication by *Blood* in April 1985 and published in October 1985.

During that period, the conclusions of the study would have been known to the other 27 participants, who included the directors of major haemophilia and transfusion centres in France, distinguished immunologists and virologists, Professor Luc Montagnier of the Institut Pasteur among them. The report was sent to the director of the French National Blood Transfusion Centre (CNTS) and was the basis of Allain's written advice, in January 1985, to the director and president of CNTS that heat treatment of blood products should begin without delay. But the paper in *Blood* also reveals the continuing uncertainties at that time. Thus the abstract of the paper concludes:

This study has shown the prominent role of LAV in the occurrence of immunological disturbances in multi-transfused patients. However, allogenic or altered proteins present in factor VIII but not in factor IX concentrates seem to play a role of immunocomprising agents. The interplay between LAV and additional factors possibly leading to acquired immuno-deficiency syndrome remains to be analysed.

Although Allain's advocacy of heat treatment had by then been supported elsewhere, doubts about the heat treatment were also prevalent; thus Bird *et al.* stated in January 1985 (*Lancet* i, 162–163) that: . . . there is therefore a considerable danger that the unproven benefits of heat treatment

will be offset by potential risks, one of which — antibody-inhibitor formation — would be irreversible.

In February 1985 there was further preliminary but clear evidence that heat treated factor VIII did not cause seroconversion.

Thus the debate about the heat treatment of antihaemophilia preparations was already in the public domain in science; it is wrong therefore to draw, as you do, an analogy with "an employee of a defence research establishment who knows that some new weapon is unsafe".

On Allain's professional responsibility, as a practising physician he was responsible for a small group of haemophiliacs, none of whom seroconverted in 1985. The knowledge that informed his own clinical practice had already been made available, as I have explained, to others with similar responsibility to patients.

Moreover, Allain had no responsibility for national strategy; he was in the third tier of administration in the CNTS, with responsibilities for a research section. His actions in alerting the administration are a matter of public record, as are those of his wife, Dr Helen Lee. For example, in April 1985, she expressed their views forcefully to more than 200 of France's haematologists. The Royal College of Pathologists' working party on the Allain affair concluded:

. . . in the months following the writing of his letter and until the universal introduction of heated factor VIII was imminent, he and his wife did all that could be expected of them to persuade those in administrative charge of CNTS to follow their advice.

It is clear that in the early part of 1985, there were still doubts in France, and that those responsible for national policy could draw attention to deficiencies in Allain's arguments. Some interpreted his advice as a panic reaction (which also happened elsewhere, in Canada for example).

For my part, I endorse the view of the Royal College of Pathologists that "it is wholly inimical to the pursuit of medical research and to the high standards of medical practice" that physicians can be prosecuted and convicted because their advice is not followed.

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Lorenzo's oil

SIR — Fred S. Rosen's review of *Lorenzo's Oil* (*Nature* 361, 695; 1993) is typical of the backlash against the film in certain sections of the medical and scientific

press. We are all, of course, entitled to our opinions. But Rosen's central reservations about the film are whether 'Lorenzo's oil' is effective in the treatment of adrenoleukodystrophy (ALD) and whether the film overstates its effectiveness. His comments in this respect demand a response.

Lorenzo's oil, the discovery of which is credited to Lorenzo's father, Augusto Odone, clearly reduces the levels of the offending long-chain fatty acids in the blood of patients with ALD. Such reduction is one of the main therapeutic goals for researchers working on the disease. What is not clear, as Rosen points out, is whether the oil prevents the onset of the disease in the target organs (the central nervous system and adrenal glands). But, given the pleiotropic effect of the ALD gene, with its variable phenotype and the disease's late onset in 25 per cent of patients, the only way in which the effectiveness of this therapy can be assessed is in a carefully controlled, long-term study (H. Moser *J. NIH Res.* 5, 35–36; 1993). In the meantime, the only hope for boys suffering from ALD is Lorenzo's oil or possibly bone marrow transplantation; even physicians who have publicly questioned the efficacy of the oil are continuing to prescribe it.

Further, Rosen is concerned that boys with ALD may develop thrombocytopaenia from taking the oil. However, W. H. Zinkham *et al.* have recently reported (*New Engl. J. Med.* 328, 1126; 1993) that there is no serious bleeding, although they do advise that platelet counts should be monitored. This would therefore seem to be a minor concern compared with the clinical course of ALD. Rosen thinks that the Odone's have not set a good example for biomedical progress. I disagree. In devising the oil and setting up the Myelin Project to accelerate research into remyelination of the central nervous system, the Odone's have shown that lay people can make important intellectual and supportive contributions to science.

At the very least, the film will help to educate the public about the medical aspects of a rare disease, raise funds for research and underline the need for animal research in developing treatments for incurable diseases. Perhaps the filmmakers should have shown a little more caution in stating the effectiveness of the oil. But regardless of whether the treatment works in the long run, the Odone's achievement should not be underestimated; they have set a magnificent example for medical progress, which few of us would be able to emulate.

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Love or money?

SIR — If I were an executive of a pharmaceutical company, I would do what is now happening almost monthly. Instead of increasing research in my own company, I would buy into a research outfit with its own source of funds. One problem is that, in the United States, as I am sure also in the United Kingdom, such research is supported by public funds.

Sandoz, for example, for an input of \$300 million to Scripps Research Institute (see *Nature* 362, 400; 1993) is not only buying into \$1.3 billion of National Institutes of Health funding during the lifetime of the Sandoz grant, but it is also buying into the millions of dollars that has gone into Scripps in previous years, which has undoubtedly formed the basis of present research at Scripps.

In other words, research is being bought up rather cheaply. In the example cited, Sandoz has in effect placed a lien on research at Scripps, preventing the results of that research from being presented to the open market. Scripps benefits, Sandoz will probably benefit, but the taxpayers will suffer.

What is to be done? One possibility is to insist, through regulation, that any company which has bought, even indirectly, into publicly sponsored research should recompense the public sponsor for research that has been the basis of any particular findings and that has led to a commercial product. Another possibility is to have those companies invest directly in the public sponsor. Think how many additional grants would accrue. Finally, the public sponsor should insist that once a private corporation has funded a researcher, no further grants should be made to that individual; a system of support that mixes profit and not-for-profit funds cannot be ethically maintained.

What we are witnessing, in the setting up of corporations by scientists, is the increasing privatization, for personal profit, of science as a public cooperative enterprise. What has become of the love of science, of research, of simple satisfaction with a job well done?

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Mammoth task

SIR — It is altogether appropriate that the recent discovery of remains of dwarf mammoths on Wrangel Island off the northern coast of Siberia (*Nature* 362, 337–340; 1993) should be announced in the year of the 250th anniversary of Thomas Jefferson's birth. In fact the paper

came to my attention on 13 April, Thomas Jefferson's birthday.

Jefferson was much interested in mammoths¹. He had mammoth bones in his collection at Monticello. In his advice to Andrew Michaud, the French botanist-adventurer whom he had recruited for an abortive exploration of the American Northwest (of that day, 1793, not the "Northwest" of Lewis and Clark, more than ten years later), Jefferson wrote: "Under the head of Animal history, that of the Mammoth is particularly recommended to your enquiries"².

Jefferson has long been derided by formal biologists for what they interpret as a naively romantic projection of palaeontology. His reputation as a naturalist is somewhat redeemed by the discovery of mammoth remains barely 4,000 years old, well within the period of written history (no writing or human remains on Wrangel Island, however).

Jefferson also long took issue³ with Georges-Louis Buffon, the French botanist, for his thesis that species deteriorated as one moved west. Buffon said that mammals (including human beings) native to the Western Hemisphere were smaller than those from the Eastern Hemisphere and less amenable to the effects of civilization. Jefferson spent several years obtaining a skeleton and a hide of a North American moose to send to Buffon⁴. Although these specimens did reach Paris, Buffon, who was terminally ill, probably never saw them⁵. Jefferson would feel redeemed further that the Old World mammoths are small. We have big ones here.

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2. Jefferson, T. to Mr. Andrew Michaud, 30 April 1793 in *Thomas Jefferson and the Story Mountains* (ed. Jackson, D.) 71–77 (University of Illinois Press, Urbana, 1981).

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4. Boyd, J. P. (ed.) *The Papers of Thomas Jefferson* Vol. 9, 160; Vol. 11, 68, 320 (Princeton University Press 1950–).

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Physics of life

SIR — John Maddox (*Nature* 363, 13; 1993) has demonstrated what can only be regarded as an extreme form of reductionism. Here, even restrained, he chastises molecular biology for a preoccupation with "enumeration" and "competitiveness", the former serving to keep molecular biology, as we are led to infer, failed of its true potential as a science. (The latter complaint is a red herring, as anyone who has ever read anything about the Super-

conducting Super Collider or similar projects will know.) If only molecular biologists were more rigorous — indeed, as Maddox clarifies, more like "physical chemists" — then their craft might prove invaluable as a means to "understanding" what's really going on in nature. But, I wish to interject, what is it that Maddox thinks is *really* going on?

Suppose we ask for the most complete explanation of any particular event, what would count as such? Maddox would surely admit that for hundreds of years there were many scientists who assumed that no complete explanation of physical events could be given without the invocation of divine power. Descartes, for example, crafted a dualism for the sake of giving what he thought was a complete explanation of the mind *and* the body. Even today, issues in the philosophy of mind and psychology turn on the question of the *level of ontology* of explanations: surely neurophysiological explanations of our wants and desires will some day be possible, but that does not mean that intentional explanations (for example, 'he took a drink because he wanted water') are any less complete and genuine as lower level physical explanations.

What we need to focus on in any level of explanation is the nearness to lawfulness that is achieved: counterfactual supporting generalizations in *any* of the special sciences should probably be considered laws (*ceteris paribus*, though they be). Hence, table-thumping reductionism, as Maddox gives, serves no purpose, instead it merely demonstrates an ignorance of the many levels of scientific inquiry — the different laws *and* ontology — that are possible. Now, are all of these levels to be explained by physics alone? I think not, though this is not a retreat to a pernicious dualism. Instead, it is to emphasize the rather hefty price one must pay for reductionism.

Poor Descartes, he is in such bad favour these days, what with all the scientific progress around. But is it really sensible to pull back all the way to the other side, to go physicalist come what may? Hardly. Molecular mechanisms across many species are indeed interesting, but surely one of the most intriguing things is to look at this the other way round: how could the same molecular mechanisms subvene for different species? I submit that the microlevel sciences (physics for example) could never answer such a question, as it occupies a place outside that domain. But then so do many other intriguing questions, like many that molecular biologists ask.

Come on, Mr Maddox, let's all pull together.

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Architecture of the invisible

John Meurig Thomas

From the structure of sodium chloride to that of a human rhinovirus complexed with its receptor — X-ray crystal analysis has taken an extraordinarily fruitful path since its inception 80 years ago.

THE philosopher A. N. Whitehead once remarked that it is more important for an idea to be fruitful than true. The idea that occurred to the 22-year-old Lawrence Bragg in 1912 — that diffraction of X-rays by a crystal is best thought of as a process of reflection from atomic planes, just like light reflected from a mirror — turned out to be both true and fruitful. Its veracity was experimentally established within a few months. And in due course it was to transform the very fabric of mineralogy, chemistry, metallurgy and biology.

Conceptually, X-ray diffraction-reflection started in November 1912 when the young Bragg described how to interpret the beautiful but complicated patterns that von Laue and his colleagues had obtained from minerals such as zinc

others 40 years earlier.

From 1913 onwards two distinct methods became available by which X-rays could elucidate the details of crystal structure. In the Laue method, polychromatic X-rays penetrate the crystal and give rise to a spot pattern on a photographic film. In the Bragg method, monochromatic X-rays of wavelength λ are reflected from a set of parallel and similar crystal planes at an angle θ when the relation $n\lambda = 2d\sin\theta$ is fulfilled (d is the distance between successive planes, θ is the glancing angle which the incident and reflected rays make with the planes and n is the order of the reflection). The set-up for the Bragg method is similar to that used in an optical spectrometer, but with an ionization chamber serving as a

intensity of diffraction peaks, nowadays require crystals with dimensions of the order of 0.1 mm^3 to yield trustworthy structural information. Twenty years ago the required dimensions were 0.5 mm^3 . But such is the intensity of synchrotron sources that samples of volume $10 \times 10 \times 30\text{ }\mu\text{m}$ — just a single grain from a powder — can now be subject to analysis.

The heroic era

In 1915, WHB proposed¹ the far-reaching idea that the periodic repeat of atomic patterns in crystals could be represented by Fourier series. "If we know the nature of the periodic variation of the density of the medium we can analyse it by Fourier's method into series of harmonic terms . . . We may even conceive the possibility of discovering [from the relative X-ray] intensities the actual distribution of the scattering centres, electrons and nucleus in the atom". From the time, shortly after they shared the Nobel prize in physics in 1915, when they agreed that one of them (WLB) would concentrate on inorganic crystals and the other (WHB) on organic crystals, the Braggs and their increasing band of converts explored various ways in which to retrieve electron-density distributions from X-ray diffraction data. The fundamental difficulty centred on the so-called phase problem. To arrive at a three-dimensional map of electron density within a crystal it is necessary to know the phase and the amplitude of the Fourier components. But the phase is lost in recording X-ray diffraction data.

In a monumental paper² on the silicate mineral diopside ($\text{CaMg}(\text{SiO}_3)_2$), published in 1929, WLB calculated the two-dimensional Fourier projection which revealed the positions of the Ca, Mg, Si and O atoms. Max Perutz has drawn attention to the enormous arithmetic labour involved in this work: a total of 17,760 numbers had to be summed, and this WLB did single-handedly. This was the beginning of the method of Fourier projections which was used to solve hundreds of crystal structures in the succeeding 30 years, until the advent of digital computers made it possible to calculate Fourier series in three dimensions. In the mid-1930s Robertson and Woodward³, working on crystals of the phthalocyanines, successfully demonstrated the heavy atom (isomorphous) replacement method for overcoming the phase problem, thereby solving the complete molecular consti-

IMAGE
UNAVAILABLE
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REASONS

Crystallographic pioneers — Max von Laue flanked by Lawrence Bragg (left) and William Henry Bragg (right). Credit: Science Photo Library.

blende. But the genesis of X-ray crystal analysis, with its ability to reveal the structure of solids in atomic detail, occurred in 1913 with the publication of two papers in the July issue of *Proceedings of the Royal Society*. One was by W. L. Bragg (WLB) alone on "The Structure of Some Crystals as Indicated by their Diffraction of X-rays", the other by WLB and his father W. H. Bragg (WHB) on "The Structure of Diamond". Both shock and exhilaration greeted the publication of these papers. Shock because it was incontrovertibly established that there was no molecule of sodium chloride inside rock salt, simply an extended alternation of sodium and chloride ions. Exhilaration, because the structure of diamond confirmed the tetrahedral coordination of carbon as envisaged by van't Hoff and

detector. Because the results were more readily translated into structural information, and also because the Braggs (WHB especially) were dextrous in the use of ionization detectors, the Bragg method was usually preferred over the Laue one. Later, photographic moving-film methods displaced the early cumbersome detectors until they in turn were displaced by computer-controlled diffractometers. Recently, however, especially since the advent of synchrotron radiation and of area detectors, the Laue method has been resurrected. For certain problems such as charting dynamic changes within crystalline enzymes, it is more convenient than the Bragg procedure. Conventional (or rotating-anode) laboratory X-ray sources, employing automatic and computerized detectors for recording the position and

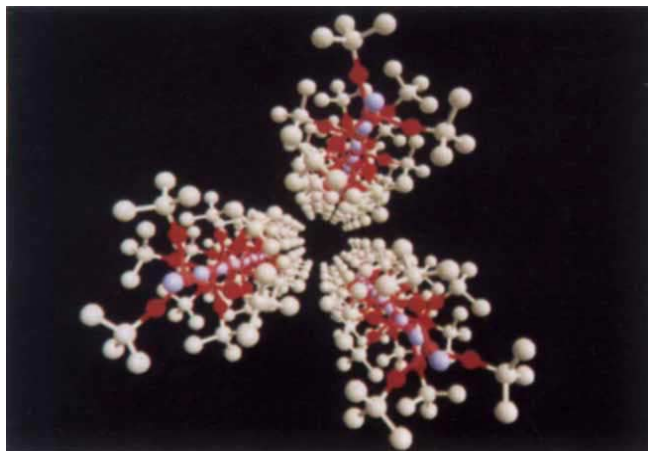


FIG. 1 Crystal structure of a metallomesogen (metal-containing liquid crystal). The linearly bonded atoms of tungsten confer remarkable properties on new types of liquid crystals. Courtesy of M. H. Chisholm and J. C. Huffman.

tution and three-dimensional structure of the metal-substituted and metal-free phthalocyanines.

The study of alloys in the 1920s led to the structures of γ -brass and α -manganese and the production of a series of alloy phase-diagrams using the powder method (introduced earlier by Debye and Scherrer). Hume-Rothery's famous electron-atom ratios emerged from the knowledge so gained: he established that ratios (or electron concentrations) of $\frac{1}{2}$ ($2^{1/4}$), $\frac{2}{3}$ ($2^{1/3}$) and $\frac{3}{4}$ ($2^{1/2}$) were characteristic of structures of body-centred cubic, γ -brass and close-packed hexagonal types respectively. This fact was later to receive a quantum mechanical explanation in terms of Brillouin zones, and alloy studies generally were to lead to the brilliant theory of order-disorder transformations in alloys by E. J. Williams⁴.

In the early 1950s Karle and Hauptman⁵ registered a signal advance when they showed how structures could be solved from X-ray diffraction by direct methods. The phase problem could be tackled mathematically. Nowadays the structures of many complicated molecules may be solved directly from the intensities of the X-ray pattern of the corresponding crystals. Often, however, a major obstacle in structural elucidation is obtaining a suitable crystal for analysis.

It is interesting to note that the proposed buckminsterfullerene structure for C_{60} remained no more than an interesting speculation from 1985, when C_{60} species were detected in the gas phase, until 1990 when C_{60} was isolated in macroscopic quantities⁶. A further year elapsed before X-ray analysis of a heavy-metal derivative of C_{60} established its now well-known, truncated icosahedral structure⁷.

The inorganic world

In notes bequeathed by WLB to Perutz⁸ he writes "The analysis of diopside was a turning point in our ideas about silicate

structures. I showed that the 'SiO₃' which appears in the chemical formula does not represent SiO₃ acid groups but a string of SiO₄ groups joined by shared oxygen atoms. It was a crucial step in showing that silicon occurs in a tetrahedral group of oxygen atoms". The profound impact of X-ray analysis on Earth science as well as inorganic and solid-state chemistry, solid-state physics and materials science at that time may be gleaned from one of

the most significant chemical texts published (in 1939) this century, Linus Pauling's *The Nature of the Chemical Bond and the Structure of Molecules and Crystals*. More so than WLB, Pauling realized that, with inorganic structures, it was profitable to identify polyhedral units around a central 6- or 4-coordinated ion. Pauling, WLB and Goldschmidt each saw the merit of deducing ionic — and covalent (metallic) — radii from crystal structures and they helped lay the foundation of crystal-field theory and subsequent advances in valence theory. Pauling saw the ubiquity of isomorphous substitution in the mineral kingdom, especially in the enormous range of new silicate and aluminosilicate structures that had by then been discovered. Recognizing the observed propensity for ions such as Li⁺ to replace Mg²⁺ and Al³⁺ to replace Si⁴⁺, Pauling brilliantly analysed and interpreted the major categories of silicate minerals into their chain (1-D), sheet (2-D) and network (3-D) forms. This achievement still exerts a profound influence in geochemistry and in materials science and engineering.

There were also some surprises. Thus, whereas in the gas phase and in solution PBr₅ and PCl₅ exist as discrete molecules, it transpired that, in the crystalline state, the former occurs as PBr₄⁺Br[−] the latter as PCl₄⁺PCl₆[−]. (Very recently X-ray analysis has unearthed a new, metastable form, (PCl₄⁺)₂PCl₆[−]Cl[−].) And whereas in the oxides of certain transition metals such as NiO, point defects were found to be present both because of discrepancies between X-ray and pycnometric densities and from unit cell dimensions, Magneli⁹ found that, in grossly non-stoichiometric tungsten oxide, WO_{3-x} (0.0 < x < 0.5), there was no need to postulate the existence of any point defects. Removal of oxygen by reduction from WO₃ generates so-called 'shear planes' in which the normal corner-sharing of WO₆ octahedra changes to edge-sharing, and this occurs in

a crystallographically well-defined family of planes, just as postulated¹⁰. (Homologous series of non-stoichiometric oxides, for example W_nO_{3n-1}, W_nO_{3n-2} and so on (with *n* being typically 10, 11 . . .) were later discovered by high-resolution electron microscopy — see later.)

Much new light has been shed on the bonding within and the overall electronic structure of inorganic solids, and attempts have been made to determine the ionicity of compounds from their X-ray reflection intensities. And by simply noting the precise separation of one metal atom from another in ionic entities or extended arrays within solids, decisions are readily made as to whether there are single or multiple bonds formed between them (Fig. 1).

In the realm of transition-metal-organic complexes, dramatic revelations came from the X-ray crystal analysis of vitamin B₁₂ by Hodgkin¹¹. Until 1964 it was thought that compounds with Co-C σ bonds should be unstable and reactive, if at all capable of existence. Her discovery that a cobalt enzyme of vitamin B₁₂ contained an adenosyl group linked to cobalt

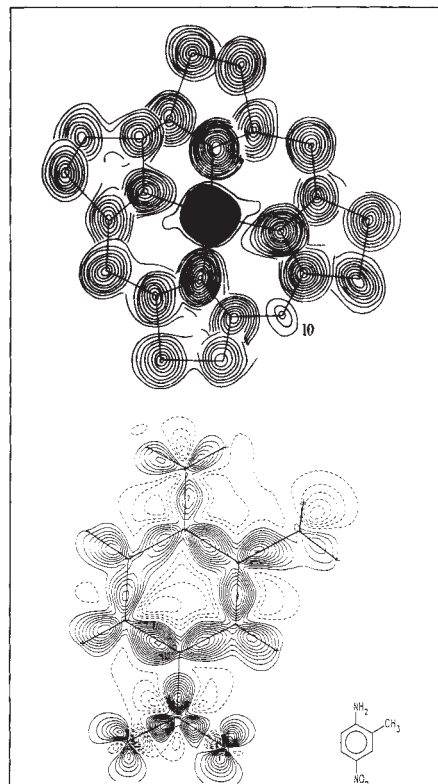


FIG. 2 Top, electron density distribution as calculated by Hodgkin and colleagues for the essential core of a vitamin B₁₂ derivative. The cobalt atom is clearly visible. Courtesy of Royal Society. Bottom, deformation density map of the nonlinear optical material 2-methyl-4-nitroaniline. Note the presence of density in bonding regions between atoms and in the lone pair regions, as around the nitro group oxygens. Courtesy of M. B. Hursthouse.

by a direct Co–C σ bond was, therefore, rather surprising (Fig. 2). Her work on vitamin B₁₂ brilliantly established the constitution, configuration and conformation of the molecule, the asymmetric unit formula of which is C₆₃H₈₈N₁₄O₄PCo. At the same time it revealed a structural motif, the so-called corrinoid ligand system, not previously encountered in natural product chemistry.

In the burgeoning field of metal cluster and organometallic chemistry, notwithstanding the recent advances in structural techniques such as fast atom bombardment mass spectroscopy and the myriad manifestations of nuclear magnetic resonance, X-ray crystal analysis (occasionally aided by neutron analysis) reigns supreme. As well as various types of static and fluxional polyhedral arrangements adopted by clusters of metal, draped with stabilizing moieties such as carbonyl or triphenylphosphine (PPh₃) groups, insights have been gained into the bonding of light elements to these metals. This has contributed to our understanding of the catalytic properties of metals such as platinum, ruthenium and cobalt. The fact that

understandable in terms of the strong three-dimensional sp³-bonding of its constituent atoms, the low frictional properties of graphite are not attributable to the loosely bound layers of sp²-bonded carbons, because, in a vacuum, graphite is a very poor lubricant. In general, it is the crystalline defects, especially dislocations which are not detectable by conventional X-ray analysis, rather than the X-ray-derived ideal structures, that hold the key to the mechanical behaviour of solids. The same can be said of the luminescent properties of solids, but not necessarily so of optical, dielectric, ferroelectric and piezoelectric properties since these can be divined from the dynamic (temperature-dependent structural) attributes of a solid.

Organic molecules

In 1928, there came an early triumph for X-ray crystallography in organic chemistry when Kathleen Lonsdale showed that the aromatic ring in crystals of hexamethylbenzene was planar, the first direct proof that this was so. Later, Robertson and his school explored numerous polynuclear aromatic hydrocarbons and showed that many of the rings in such compounds are not regular. The results posed new challenges to theoreticians concerned with establishing the relationships between observed bond length and bond order.

Hodgkin's work¹¹ on cholesteryl iodide, where the halogen served as the heavy atom for convenient phasing, confirmed the sterol formula proposed earlier by natural product organic chemists. But when she solved the crystal structure of penicillin, she moved ahead of the organic chemist. By ingenious biosynthetic methods a heavy atom such as bromine could be introduced, making it easier to solve the structure of the resulting *p*-bromo-phenoxymethylpenicillin¹¹.

Gradually enormous numbers of structures were solved, embracing terpenes, alkaloids, polysaccharides and a number of synthetic polymers of several distinct kinds¹². Using anomalous scattering, it became possible, thanks to the work of Bijvoet, to determine the absolute configuration of chiral molecules. It was shown for the first time that the conventional configuration for the D-tartaric molecule as proposed by Emil Fischer 60 years earlier did in fact correspond to reality. By 1960, X-ray crystallography

began to oust degradative organic chemistry as the main source of information on the structure of natural products. The number of complete structure analyses (organic and organometallic) mentioned in the Cambridge Crystallographic Database was a mere 8 in 1945, rising to 118 in 1960; it now runs at the rate of about 10,000 per year, and the present total is over 100,000.

The systematic study of related organic compounds, such as the amino acids by the Caltech group, led Pauling and Corey¹³ to propose the α -helix structure for polypeptides: this was later confirmed by X-ray crystallography. It is ubiquitous in proteins. In 1940, Pauling and Debrück, guided by observed architectural patterns in crystals, proposed that a molecule serving as template would favour the production of another with complementary structure. This is now an important principle in the design of catalytic antibodies and new molecular receptors.

Gradually it emerged that polymorphism among organic crystals is the rule rather than the exception; it also transpired that molecules that are photolabile in the dispersed state may be rendered relatively inactive when they adopt certain packing motifs within a molecular crystal. Contrariwise, certain molecules that yield a given set of products when photostimulated in solution or melt, may produce a highly stereospecific product in a crystal that has been fashioned to order by crystal engineering¹⁴. This is a fast-growing field, which, by recognizing the dynamics of atomic and molecular movements within crystals, serves as a basis for the production of (lightweight and strong) non-linear optical, photochromic, ferroelectric or pyroelectric materials for molecular electronic devices. The design of crystals with desirable chemical and physical properties is aided by crystallographic databases. They reveal 'steering' devices, such as C–H...O bonding or intermolecular–Cl...Cl– attraction, which can be harnessed to advantage for the production of prochiral monomers for use as pharmaceuticals or novel sensors. Such databases are also a source of insights into intermolecular reaction pathways¹², permitting quantitative computer simulations of processes such as nucleophilic or electrophilic substitution. All this has made it possible¹⁵ to devise a stereochemical methodology for control of the growth, dissolution and morphology of crystals.

Biology

The pioneers of X-ray analysis were greatly surprised to discover that many of the constituents of the living world (apart from bone and teeth) were partially or wholly crystalline. There had been an ingrained belief that macromolecular entities, such as enzymes, nucleic acids, polysaccharides and fragments of tendon

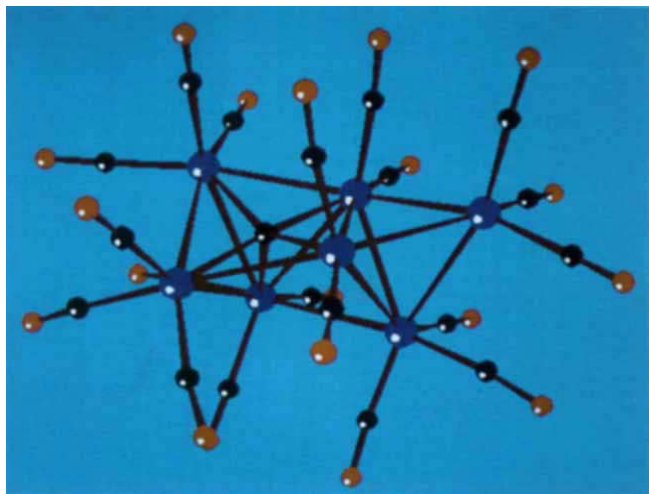


FIG. 3 Structure of Os₇ carbido clusters. Osmium atoms are coloured blue. Courtesy of P. R. Raithby and J. Lewis.

stable Pt–H, Pt–C, Pt–N and Pt–O bonds freely occur in certain organometallic complexes also accounts for the versatility of metallic platinum in catalytic conversions of a wide range of organic compounds. X-ray analysis shows that some hexametallic clusters, in which the metal atoms are at the vertices of an octahedron, accommodate a central carbon in 6-coordination (as in [Co₆C(CO)₁₄][–]) (Fig. 3). This compound, just like Ru₆H(CO)₁₈ where the H is also at the centre of the octahedron, reveals a new kind of bonding between metal and non-metal.

Early hopes that knowledge of the atomic architecture of inorganic materials would deepen understanding of their mechanical as distinct from their chemical properties have not in general been fulfilled. Although the hardness of diamond is

and muscle would be at best colloidal, and therefore amorphous. Not so. Astbury's X-ray work in the mid-1920s on wool, hair and silk showed that these materials were essentially crystalline. And when, in the mid-1930s, Bernal and Hodgkin found that 'wet' globular protein crystals such as the enzyme pepsin yielded sharp X-ray diffraction patterns which extended to spacings of the order of interatomic distances, it inspired Perutz to embark on his path to glory in elucidating the structure of haemoglobin. Astbury in 1945 demonstrated the α -, β - and supercontracted states of keratin-like fibrous proteins; and Herman Mark contemporaneously produced the first interpretation of the X-ray pattern of crystalline cellulose.

These were stepping stones on the road to the breakthrough of 1953, when Watson and Crick¹⁶ interpreted Franklin and Wilkins's data on DNA in terms of the renowned double helix — an advance which, at a stroke, offered both an explanation of the function of the molecule and opened up the molecular basis of genetics and innumerable other facets of the science of living things.

Whereas DNA yielded its secrets through X-ray data acquired from fibrous materials, the globular proteins, haemoglobin and myoglobin (in the hands of Perutz and Kendrew respectively) came from beautifully crystalline materials. It was chiefly the heavy atom (isomorphous substitution) technique for solving the phase problem that proved to be the crucial factor here. Fortunately, complex molecules such as haemoglobin take no more notice of the isomorphous attachment of a heavy atom than (to use WLB's words) "a maharaja's elephant would of the gold star painted on its forehead". Myoglobin contains some 2,500 atoms, and haemoglobin is four times as large — a far cry from diopside and rock salt. The labour involved in crystal harvesting, collection, correction and processing of X-ray intensities from hundreds of thousands of reflections was enormous. But the rewards were immense. Not only did Perutz's work reveal the marked structural change accompanying the reaction of haemoglobin with oxygen, it heralded a new era for adventures in enzymology, virology and other branches of biology.

In the mid-1960s, D. C. Phillips was the first to solve¹⁷ the structure of an enzyme (lysozyme), and in doing so he clarified its mode of action. This act of explaining the function of an enzyme, by determining its structure in the presence and absence of bound substrate, marked a turning point in enzymology. The cleft in the enzyme's architecture, the lining of which serves as the active site, could be specified in atomic detail by Phillips. Since then, the architecture of numerous other enzymes has been worked out, and there is now a

growing database of enzyme structures at Brookhaven. Of late, by deploying either laboratory (Bragg monochromatic) or synchrotron (Laue polychromatic) methods, the kinetics of enzymic catalytic conversion have been charted¹⁸. X-ray data from a protein crystal can be measured in 100 picoseconds using synchrotron radiation. The enzyme haloalkane dehalogenase, one of the so-called α/β hydrolase fold proteins which are thought to have evolved from a common ancestor, had its amino-acid sequence determined by X-ray crystallography in 1991. Very recently¹⁹ the enzyme has been subjected to a time-lapse (crystallographic snapshot) study as a function of pH and temperature, and it has been demonstrated that catalysis proceeds by a two-step mechanism involving an ester intermediate. This study is rather in the fashion of the classic X-ray work (by difference Fourier analysis) of the catalytic breakdown of transfer RNA by Pb ions²⁰, which serves as a model for the role of metal ions in ribozymes. With site-selective mutagenesis, and the sophisticated techniques of present-day synthetic organic chemistry, it is now possible to design modified as well as miniature versions of many enzymes and to study them *in situ* by X-ray analysis. In 1986, for instance, a miniature 'chymotrypsin' was synthesized, one tenth of the size of the real one, by grafting the triad of amino-acids of the active site on to the rim of a cyclodextrin²¹. Its performance rivalled that of chymotrypsin itself.

But the structure of an enzyme does not always disclose the secrets of its mode of action. This is an extra reason why time-resolved studies, aimed, among other things, at capturing active intermediates, are now pursued with such zeal. Synchrotron radiation can now be deployed in a combined X-ray diffraction and X-ray absorption mode, as shown for inorganic systems²², so prospects look bright for *in situ* diffraction-absorption studies of a wide range of enzymes and related materials.

Two of the biological triumphs for X-ray crystal analysis are the elucidation of the three-dimensional structure of a photosynthetic reaction centre²³ (which required determination of 36,000 atomic parameters), and that of a virus-cell receptor complex. For the former, X-ray analysis alone proved adequate, but for the latter high-resolution X-ray analysis (via monochromatic synchrotron sources) had to be supplemented by relatively low-resolution electron crystallography. Knowledge of the structure of viruses generally owes a great deal to the work of Klug and colleagues. In 1968, they introduced²⁴ a new Fourier-based method that uses the symmetry of helical and icosahedral viruses to produce three-dimensional reconstructions of simple

viruses based on two-dimensional electron micrographs of stained specimens. Fourier electron microscopy takes advantage of the large depth of focus of an electron microscope, and of the fact that all features along the direction of view of the specimen are superimposed in the image. This ingenious development entails Fourier transformation of the digitized image — in which phase as well as amplitude information is retained — thereby yielding, after due correction, a planar section of the diffraction pattern of the object under investigation. When a number of such planar sections are combined it is possible to construct a complete three-dimensional sampling of the diffraction pattern by interpolation and then, by inverse Fourier transformation, to construct a three-dimensional image of the object. In the past 20 years these techniques have provided a wealth of detail on the molecular structure of several viruses, phages, organelles and muscle filaments.

In 1975, Henderson and Unwin²⁵ extended these techniques to unstained

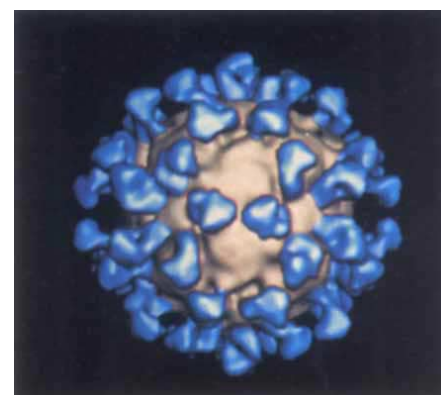


FIG. 4 Human rhinovirus complexed with Fab antibody fragments (blue). Two Fabs cross over the icosahedral twofold axis and bind bivalently. Courtesy of T. S. Baker and N. H. Olson.

specimens of two-dimensional crystals embedded in glucose and showed that high-resolution internal details could be imaged. Using electron crystallography Henderson and co-workers have solved²⁶, at a resolution of 3.5 Å, the structure of the light-driven proton pump bacteriorhodopsin. Taking advantage of advances in sample preparation developed by Dubochet, Unwin has now elucidated the structure of the nicotinic acetylcholine receptor²⁷, a ligand-gated ion channel involved in signal transmission at the chemical synapse.

The combination of X-ray and electron crystallography constitutes an exciting development in molecular biology, for it can tackle the global structure of interacting components like a virus and an antibody. For instance, this combined approach has been used to derive models of rhinovirus with an Fab antibody fragment and with its cellular receptor^{28,29} (Fig. 4). One

consequence of the first of these studies is that the footprint of the Fab on the virus surface and its topography can be clearly identified: it covers an area of some $500 \text{ \AA}^2$, which is much larger than the site defined by genetic studies.

Electron crystallography

Several lines of research will continue to rely on existing tools for structural analysis; and it is likely that the marriage of X-ray and electron crystallography will prove even more productive than in the past. There are at least two exigent needs. First to develop the techniques, rooted in X-ray analysis, that, in the biological domain, will clarify the nature of receptors and elucidate the mechanisms of molecular recognition, signalling and triggering in their broadest contexts. Second, to cope with both inorganic and organic materials (as well as their composites) at the ultramicroscopic scale, involving particulate matter down to volumes of about 1 nm^3 .

The approach to the first need is already clear, as indeed it was more than a decade ago³⁰: "One studies a complex system by dissecting it out physically, chemically, or in this case [chromatin] enzymatically, and then tries to obtain a detailed picture of its parts by X-ray analysis and chemical studies, and an overall picture of the intact assembly by electron microscopy". The work of Baker and Rossmann *et al.* on viruses, and of Rayment *et al.*³¹ on the acto-myosin complex serve as contemporary paradigms for the combined approach of X-ray diffraction and electron crystallography.

Approaches to the second need entail deployment of synchrotron sources in new ways, and also taking full advantage of ultra-high resolution electron microscopy. With the highly defined tunability, monochromaticity and intensity of synchrotron radiation, powder X-ray diffraction can be taken to much greater heights.

Profile analysis using the Rietveld refinement method (first developed in 1969 for neutron diffraction patterns) can yield definitive structural data on powdered samples, such as zeolites and mixed metal oxides, that are difficult to prepare as single crystals. By using anomalous scattering, the oxidation state of ions on specific sites can be determined by methods pioneered by Cheetham and others³². Powder profiles are nowadays amenable to analyses that yield full, three-dimensional crystal structures. And *in situ*

dimensional projected structures from which the precise positions of the heavier atoms may be trustworthily read off (Fig. 5). By combining images recorded down different zone axes, the complete structure can be determined³³. Spatial resolutions of about $1 \text{ \AA}$ are now achievable; and this technique of direct imaging straightforwardly identifies recurrent or regular intergrowths, superlattices, incommensurate sublattices, nanotextural irregularities such as sub-grain boundaries or condensed aggregates of defects and

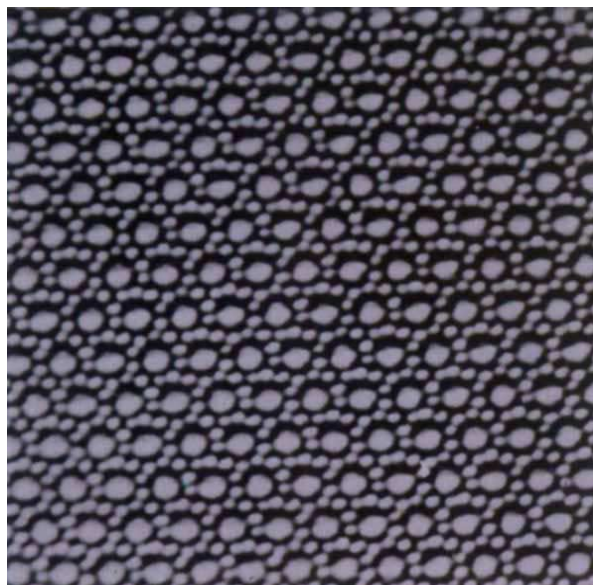


FIG. 5 Electron micrograph of a zeolitic acid catalyst, showing projected structure of pores lined with active sites. Large pores are $5.5 \text{ \AA}$ in diameter. See *J. M. T. Sci. Am.* 266, 85–89 (1992). Courtesy of P. L. Gai-Boyes and J. M. T.

combined X-ray diffraction and X-ray absorption measurement that chart long- and short-range order, respectively, are already a reality, thanks to the availability of position-sensitive detectors and photodiode arrays³².

Real-space high-resolution transmission electron microscopy for those inorganic materials that are undamaged by intense electron beams directly yield two-

impurities. Moreover, electron-induced X-ray emission and electron-energy loss spectra may now be routinely recorded simultaneously along with ultra-high resolution imaging, and all this on individual particles of less than a femtogram³⁴. New structural types of solid catalysts, new kinds of molecular sieve adsorbents, new modes of discontinuities with ceramic superconductors as well as the crystalline order within $50 \text{ \AA}$ particles of metal colloids of the kind prepared by Faraday 140 years ago, have been uncovered by this microscopic approach to crystal analysis.

Statements such as Dorothy Hodgkin's when she received her Nobel prize for X-ray crystallography — "I seem to have spent more time not solving structures than solving them" — will be less valid in future. And one cannot help reflecting upon how wrong-headed the eminent biochemist Sir Ernst Chain was when, 20 years ago, he advised the British Prime Minister that medical research involving crystal structure analysis was a profligate waste. □

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Paper crystals of molecular hydrogen and ice

The complexity of solid structures containing hydrogen bonds should be a salutary reminder that there is a long way to go before some of the simplest solid structures are understood.

It is not surprising that the simplest materials, bulk water and molecular hydrogen in particular, are among those whose bulk structures are the most perplexing: the mass of the hydrogen atom is only a third of that of the next heavier atom capable of forming molecules with ease. And even lithium can be a nuisance, as the long saga of attempts to calculate the structure of molecular lithium hydride attests. (Is it a molecule, or a complicated kind of atom?) Low mass, of course, implies both mobility in the classical sense and also the need to take account of the quantum properties of protons, as in the ubiquitous hydrogen bond.

Yet the arrangement of molecules in the bulk structures of these materials is of great practical importance. Liquid water is the most common of all solvents, but its versatility is still better explained by hand-waving than by calculation. Molecular hydrogen is a different kettle of fish. If hydrogen is ever widely used as a transportable fuel, perhaps as a substitute for petrol or gasoline, there will no doubt be an urgent need for an accurate description of its equation of state in a form that automobile engineers can use. Meanwhile, the equation of state of pure hydrogen at pressures greater than those likely ever to be allowed on the public highways is of great concern to those who worry about the internal constitution of stars and even planets.

It is not really such a long time ago, a mere 40 years, since the late W. H. Ramsey set then-infant planetary science back on its heels with the opinion that the crude data on the mass and density of the planets could best be explained if the material at their core were hydrogen converted into metallic form (necessary to account for the Earth's magnetism) by pressure. In the event, the entertaining theory was undermined by the accumulation of better data, and has since been made entirely untenable by calculations of the transition of hydrogen to the metallic state. But who knows what happens at the centre of Jupiter or Uranus?

The problem with molecular hydrogen is easily appreciated. Suppose you arrange that hydrogen molecules are laid end to end along a line in such a way that the spacing between one end and the next is equal to the intramolecular spacing of the two protons. The result is a one-dimensional lattice with a proton on every site. The structure thus formed, by whatever magic, will not be a mere assemblage of molecules, but a unified structure in which the electrons are distributed over the entire lattice, which becomes

possessed of a well-defined band structure. By promotion of electrons from the energetically lowest (and full) band, the material would behave as a semiconductor. The structure could alternatively be described as an infinite array of hydrogen molecules at least partly cemented by mutual hydrogen bonds.

The guess prompted by that simple-minded argument is that, if molecular hydrogen is compressed, long before the average distance between molecules approaches the intramolecular separation of the protons (1.44 atomic units, say 0.07 nm), the formation of hydrogen-bonded structures will bring about departures from the behaviour of a perfect gas. In liquid molecular hydrogen, the same phenomenon should play a still more dominant role. Yet it is also known that molecular hydrogen solidifies at 14K, forming a hexagonally close-packed molecular solid in which molecules are separated by 3.13 a.u. That solid phase remains intact up to a pressure of 38 GPa, under which conditions the volume of the solid phase is reduced by a factor of more than five.

But what happens under more extreme conditions? Nobody is sure, which is why D. Hohl (from the Institute of Solid State Physics at Jülich) and V. Natoli, D. M. Ceperley and R. M. Martin (from the University of Illinois at Urbana-Champaign) have carried out a fiendishly complicated molecular dynamics study of the high-pressure regime (*Phys. Rev. Lett.* **71**, 541; 1993). In doing so, they go the whole hog, treating the protons and electrons in the structure as quantum objects. To make the computation feasible, they have supposed that their bulk material is built by the repetition of basic cubes each containing 64 hydrogen atoms. The densities at which the simulations have been run correspond roughly to pressures of 35, 150 and 300 GPa.

The fact that the study shows that decreasing molecular volume induces the expected formation of linear chains of hydrogen molecules does not detract from its interest and importance. Because molecular dynamics allows snapshots of the molecular configuration to be extracted, it is possible to confirm that zigzag chains of hydrogen-bonded atoms are formed and then are arranged in planes as the pressure is increased. But the surprise is that, even at 300 GPa, the molecular structure of what is then a solid material somehow persists. The authors promise a more detailed account of their calculations which should throw light on how the electrons behave.

The same problems arise with water in its

solid phases, but more tangibly. The structure of ice has been exhaustively studied by crystallographers for half a century. (The late Dame Kathleen Lonsdale, at the Royal Institution in London towards the end of the Second World War, used to make her measurements on ice in January and February, hoping to preserve her crystal intact by keeping the laboratory window open, to the great discomfort of those around her.) Now, no fewer than a dozen solid forms of ice are known. The phase diagram is reasonably well understood, but hydrogen bonding is again the enemy of tidiness; many of these solids are inherently disordered structures except at the lowest temperature. Whether the versatility of hydrogen bonding accounts for the multiplicity of phases seems to be an open question.

The conceptual difficulty presented by a complicated phase diagram is that of understanding how one phase is derived from another by the displacement of atoms in the first structure by definite amounts. Three authors from the University of Amiens, V. P. Dmitriev, S. B. Rochal and P. Toledano (the first two "on leave" from the University of Rostov in Russia) have now tackled that formidable problem for the various forms of ice (*Phys. Rev. Lett.* **71**, 553; 1993). One of the obvious difficulties in several of the low-pressure forms of ice is that crystalline disorder involves not merely the flipping of the allegiance of hydrogen atoms from one oxygen to another, but the presence of lattice sites that could be occupied by oxygens, but which often are not.

What Dmitriev and his colleagues have managed is to identify a body-centred substructure in unit cell of hexagonal ice (the form stable at ordinary pressure, and from which snowflakes are formed), and to show that at least nine of the 12 known forms of ice can be derived from this by specified coordinated displacement of the atoms. It is an intricate exercise of the reader's three-dimensional imagination. At least one of the forms of ice not included in the analysis (ice X) is understandably left out; its structure has not yet been determined.

The interest of this exercise is that, by showing that there are relatively simple ways of turning one phase into another next to it in the phase diagram, that model is made the more convincing. But in the process, the question of how the ordering of the oxygens and of the hydrogen bonds in these structures is at the same time made more clamant. That is where the theoreticians of ice structures should turn next.

John Maddox

Polymer tubules prepared

Angel E. Kaifer

THREAD several cyclodextrins (CDs) onto a single polymer chain, and they efficiently self-assemble. Harada and co-workers have taken advantage of this to build tubular polymeric structures. On page 516 of this issue¹, they show how last year's molecular necklace² becomes this year's molecular tubing, preparing supramolecular complexes in which a series of α -CD molecules are almost perfectly aligned. Their latest finding is that one can use the reactivity of the CD's terminal hydroxyl groups to establish covalent bonds among them, so that the individual receptor cavities combine to form a long polysaccharide-based tube about a nanometre across.

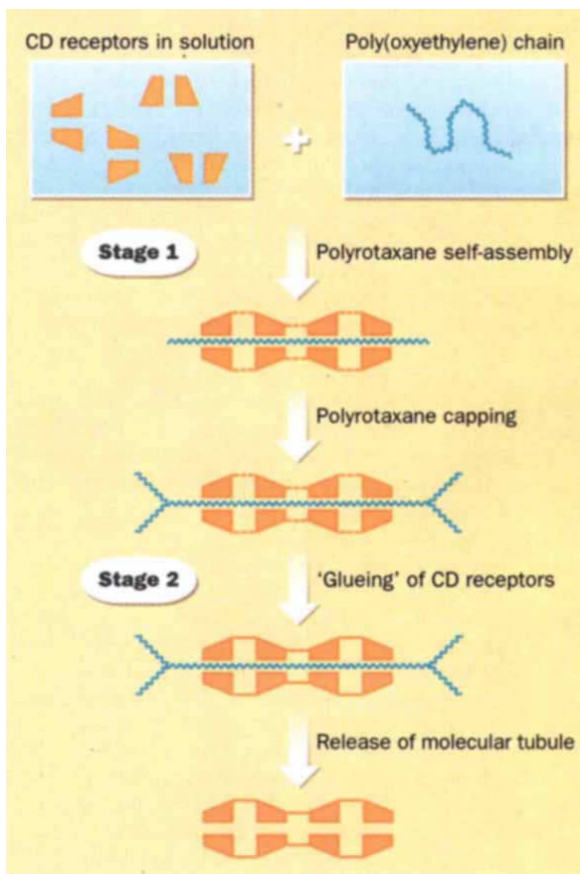
The cyclodextrins, which are composed of sugar units, are a class of natural receptor molecules without a known natural function. But their ability to bind organic substrates in aqueous media, coupled with a shape very suggestive of a lampshade, has fascinated chemists for decades. Among other applications, chemically modified CDs have been proposed as artificial enzymes.

From the smallest cyclodextrin (α -CD) come the rotaxanes, an intriguing class of supramolecular complexes in which one or more cyclic components (the beads) are threaded by a linear subunit. Bulky terminal groups prevent the escape of the beads. The cavity diameter of α -CD (~ 5 Å) is ideal for threading on extended aliphatic $[-(\text{CH}_2)_n-]$ or ethyleneoxy $[-(\text{CH}_2\text{CH}_2\text{O})_n-]$ chains, and several rotaxanes containing only one trapped bead have been made³⁻⁸. The groups of Harada² in Japan, and Wenz⁹ in Germany, last year expanded the idea to the preparation of polyrotaxanes in which numerous α -CD units are trapped after appropriate capping of the chain ends.

In Harada's polyrotaxanes², the threaded CDs are well packed, essentially covering the entire length of the polyethyleneoxy chain. The stopper groups at the ends of the chain keep the CDs in close contact with each other, as well as preventing their dissociation. The assembly of such structures is probably assisted by extensive hydrogen bonding among the $-\text{OH}$ groups that line the CD cavity openings. At this point, the tubular structure is already in place; one only needs to 'glue' each CD to its two neighbours in the polyrotaxane, remove the

stopper groups and extract the threading chain, to make the molecular polytubes (see figure).

This is precisely what Harada and co-workers have accomplished, using epichlorohydrin in basic medium as the chemical glue. In the presence of epichlorohydrin and base, the CD hydroxyl groups react to form covalent bonds between



Building designer polymer tubes out of cyclodextrin units. In stage 1, dotted lines represent hydrogen bonds; in stage 2, these are replaced by covalent bonds between adjacent CDs.

neighbouring units. A larger excess of base leads to the removal of the capping groups and to the subsequent release of the tubular CD-based polymers¹.

Molecular self-assembly is crucial to the success of this approach. The polyrotaxane structure, which can be considered as the precursor of the tubular polymer, essentially assembles itself, and the threading chain provides a support along

which the α -CD units can develop favourable hydrogen bonding interactions. Capping of the polyrotaxane keeps the aligned CD units in place, ready to undergo the cross-linking reaction that yields the tubular polymer. Obviously, polymerization of freely dissolved α -CD would not produce these remarkable tubular structures. Harada and co-workers are pioneering the use of molecular self-assembly followed by chemical reactions for the construction of nanometre-sized structures of controlled shape and size.

These hollow, cylindrical polymers are reminiscent of fibrillar or tubular structures found in living systems. But they are singularly small; their internal diameter is about 5 Å and their external diameter about 15 Å. Even the outer diameter is smaller than the average external diameter of the DNA helix (20 Å). Other relevant biological structures, such as microfilaments and microtubules, are much larger, with external diameters of 70 and 250 Å, respectively. The development of similar procedures for the organized polymerization of the higher CDs, β -CD and γ -CD, would give us tubular polymers with internal diameters of 6.5 and 8 Å. The length of the molecular tubes could probably be controlled by adjusting the length of the polymeric chains that template the formation of the precursor polyrotaxanes.

'Designer' polymeric tubes should be welcomed in separation technology, as they could be used in the manufacture of chromatographic or filtration media containing pores of well-defined molecular sizes. These structures may also serve as microreactors, a sort of organic zeolites (see the News and Views article by Davis¹⁰) with, perhaps, catalytic properties for a number of reactions. The reactivity of molecules included in these tubular polymers is expected to differ considerably from that observed in homogeneous media.

Without a doubt, these novel structures pose many research challenges; they constitute an excellent example of what supramolecular chemistry can accomplish. □

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Running out of control

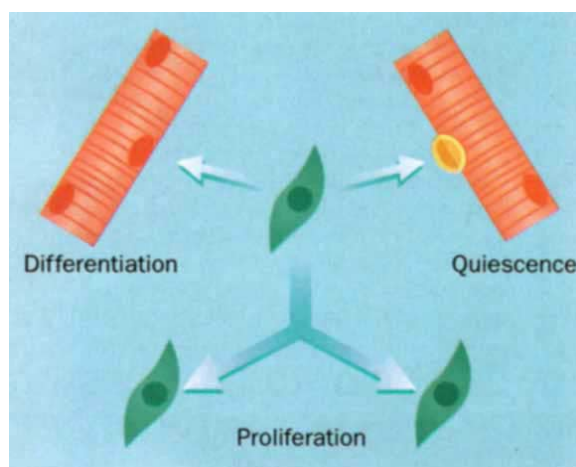
Simon M. Hughes

THERE is a Chinese proverb that says: "When the dust settles you will see if you are riding a horse or an ass". The dust was in the air when mutations of *myoD* and *myf-5*, two genes thought to control muscle formation, gave rise to mice with surprisingly mild muscle deficits¹⁻³. But that was eight months ago. Now, two papers on pages 501 and 532 of this issue^{4,5} begin to clear the air. They show that disruption of the *myogenin* gene, which encodes the third, and in some ways the most exciting, member of the MyoD family of myogenic helix-loop-helix (HLH) DNA-binding proteins, yields mice that have little skeletal muscle. So, unlike *myoD* and *myf-5*, *myogenin* seems to be required for efficient muscle formation.

Myogenin is exciting because, like the rest of its family, it can convert other cell types to muscle and is expressed early in myogenesis *in vivo* but, uniquely in the family, there is a strong correlation between myogenin expression and myoblast differentiation. Muscle is formed by a multi-step process in which proliferating mononucleate myoblasts first arise from the somites; a proportion of these myoblasts then migrate to limb or ventral body-wall regions where they cease division and fuse with one another to form multinucleate muscle fibres. Analysis of limb muscle from one *myogenin* mutant line shows greatly reduced numbers of apparently normal fibres that express myosin surrounded by masses of mononucleate cells⁴. These mononucleate cells may be undifferentiated myoblasts because limb tissue from the other mutant line will generate large numbers of myogenic cells when put in culture⁵. So the mutants seem to have a deficiency not of myoblast generation, but of differentiation.

If myogenin functions in differentiation, how can one explain the sparse formation of fibres? Myogenin may not be required for differentiation of all subpopulations of myoblasts (and there is strong evidence for myoblast diversity from tissue culture work). Alternatively, it may simply increase the efficiency of myoblast differentiation. Why myoblasts without myogenin seem to differentiate better in a dish than in the animal is puzzling. The absence of myogenin in the few muscle fibres that form could cause those fibres to inhibit differentiation of the surrounding myoblasts, an inhibition that can occur in mature muscle⁶. Or

perhaps attention should focus on the ability of transcription factors like myogenin to prevent cells entering a new cell cycle, in the manner of tumour suppressor proteins. When cultured in the absence of growth factors, even *myogenin*-deficient cells may be forced to cease division, and then differentiation may follow by default. But *in vivo*, where cells may have access to a rich mixture of growth factors and may therefore be much closer to re-initiating a cell cycle, the lack of myogenin may be sufficient to tip the balance in favour



During G1 a myoblast decides between at least three possible routes. Contrary to the classic model of a bimodal switch between cell division and differentiation, a myoblast in the animal may also have the option to become a quiescent satellite cell, which is thought to be the inactive stem cell within muscle. A complete explanation of myoblast behaviour will take this trichotomy into account.

of proliferation.

Such a scheme fits both with the independent actions of myogenic HLH family members in cell division and differentiation^{7,8}, and with the finding⁹ that MyoD and myogenin binding to the unphosphorylated retinoblastoma gene product (pRB) may enhance both the tumour-suppressing abilities of pRB and the capacity of myogenin to activate muscle differentiation-specific genes. Moreover, the differentiation of myoblasts can be regulated by growth factor-dependent phosphorylation of myogenin¹⁰, an observation that strongly suggests that myogenin is involved in cell-cycle control. As the details of the mechanism by which the G1 phase of the cell cycle is regulated become clearer¹¹, it seems likely that muscle, through the myogenic HLH proteins, will be at the centre of studies of how cells make the decision between proliferation, quiescence and differentiation (see figure).

Although the *myogenin* mutant mice

generated in both studies^{4,5} do have some differentiated muscle fibres, the two papers diverge about the severity of the deficit in different muscles. Nabeshima *et al.*⁵ find more extensive muscle fibre formation in back and intercostal muscle (which may derive directly from the somites, where muscle differentiation occurs before myogenin expression) than in limb and ventral body-wall musculature (which derives from somites indirectly through a population of migratory precursor cells, that only initiate detectable myogenin expression as they differentiate)¹². Hasty *et al.*⁴ do not report these regional differences. The discrepancy, if confirmed, could arise either from differences in the genetic background of the two mouse strains or, more worryingly, from minor differences in the constructs, which would imply that one (or both) animals lack true null alleles.

The *myoD*, *myf-5* and *myogenin* mutant mice have several features in common. In each case myoblasts are formed and can differentiate into multinucleate muscle fibres, so none of the genes is absolutely required either for commitment to the muscle lineage or for terminal differentiation into post-mitotic contractile muscle. These animals provide a means of examining the interactions of myogenic HLH family members by breeding for double mutants and by analysing the behaviour of cultured cells that are missing particular genes. The finding that MyoD can induce fibroblasts from the *myogenin* mutant mice to differentiate as muscle⁵, is likely to be a first step to the dissection of the regulatory loops controlling myogenesis. It seems that as the dust settles around myogenin and its kin, we may find a race of thoroughbreds that will run and run. □

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Size segregation mechanisms

G. C. Barker and A. Mehta

PEBBLED beaches, industrial powders and jars of mixed nuts have this in common: when shaken or agitated, the larger particles rise to the top. But the microscopic mechanisms underlying this segregation and the salient features of the 'shaking' processes that drive it are far from clear. Knight and co-workers have now put forward a new idea: that the formation of convective rolls in a shaken powder provides a mechanism for size segregation¹.

Much of the modern interest in segregation mechanisms was stimulated by two-dimensional computer simulations of shaking by Rosato *et al.*². In their picture, each cycle of applied vibrations causes all the grains to rise from the base of the container, so that the granular bed becomes dilated. Then, during settling, the smaller particles can fall more freely; larger grains require larger voids to fall into, and these can be generated only by the collective motion of many small grains, which is statistically unlikely. The large grains therefore appear to rise through the bed. There is no size threshold below which the segregation ceases, but an isolated impur-

ity will rise only intermittently if the size ratio of large to small particles is low (at least in finite-sized computer simulations). For large particle size ratios, the segregation is a continuous process.

The key feature of low-temperature Monte Carlo simulation techniques, such as the one used by Rosato *et al.*, is that they include simultaneous and cooperative grain movements (known as 'non-sequential' particle dynamics). Non-sequential elements are an essential feature of granular dynamics³. Crudely speaking, this is because granular configurations are not subject to the constant jostling of brownian motion, and therefore clusters of grains, once formed, are frozen in. The competition between the motion of these clusters and the motion of independent grains is crucial for size segregation.

The confusion raised by a simple model of shaking that omitted collective motion illustrates this point. The simulations made by Jullien and co-workers⁴ predicted a size threshold for segregation and provided no role for the shaking amplitude, two features which run contrary to

most of the experimental evidence⁵⁻⁷. The origin of these discrepancies can be traced to the purely sequential and deterministic granular dynamics used by Jullien *et al.* to drive segregation; more realistic behaviour is recovered only if collective effects and complex coupling of the granular assembly to the driving force are included⁸. This coupling reflects the complex fashion in which the driving force is transmitted to an interior grain through inelastic collisions, variable interparticle interactions and friction, if any; that is, the effect of tapping a tray, a jar or a conveyor belt will not be felt by all grains alike.

The experiments of Knight and co-workers add another ingredient to this complicated picture: convective motion. Using cylinders filled with glass beads 2 millimetres in diameter and also containing one or two larger beads of the same composition, they have demonstrated that, under conditions of low amplitude and high acceleration, convective motion causes size segregation in a vibrated bed.

They monitored the progress of selected (died) beads — the large beads, and the layer of small beads in which they originally nested. Both large and small particles were borne upwards along the middle of the cell, they found, but while the smaller particles were transported

Windows open onto Titan's surface

WRAPPED in a mystery inside an enigma, Saturn's moon Titan is cloaked by a dense atmosphere, making its surface the largest unexplored area in the Solar System. At visible wavelengths a thick stratospheric haze hides the surface from view, and Voyager images such as this one show virtually no features. Infrared imaging is difficult because of the large atmospheric concentrations of methane, nitrogen and hydrogen. But in the near-infrared, extinction by the haze is not such a problem, and there are a few narrow spectral 'windows' in which the atmosphere absorbs only weakly.

Caitlin Griffith, on page 511 of this issue, uses these windows to explore Titan's surface; she presents Earth-based measurements of the respective albedos when Titan is at eastern and western elongation relative to Saturn. (Although Titan's rotational period is unknown, Griffith assumes that its rotation is synchronous with its orbit around Saturn, so that opposite hemispheres face the Earth when Titan is at opposite elongations.) She shows that the albedo at eastern elongation is larger than that at western elongation

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

for two different orbital cycles.

In a separate paper, Lemmon *et al.* (*Icarus* 103, 329–332; 1993) detect a similar variation in albedos for a single orbit, and suggest that they may be observing some kind of surface feature. But as they have results from only one orbital cycle, it is hard to rule out the

possible influence of the transient clouds that can sweep across the surface. Griffith's observations for two additional cycles place the interpretation beyond doubt — Titan's surface is clearly heterogeneous.

This poses a problem for current theories about the surface composition. It is widely believed that the surface is coated with liquid methane and ethane, partly because methane has a very short lifetime in the atmosphere, so the high atmospheric concentration of methane implies a readily available source such as a surface ocean. Also, atmospheric methane photolyses to form ethane, which is a liquid at Titan's cold surface temperature. But Titan's large orbital eccentricity means that if there is an ocean, it should be global and more than 400 metres deep, which is hard to reconcile with the heterogeneity seen by Griffith. In fact, Griffith suggests that her observations are consistent with a water-ice feature on the surface. More information should be forthcoming if the Cassini mission manages to map the surface in more detail using the same near-infrared windows.

Gabrielle Walker

downwards in a thin convection zone at the cell edges, particles larger than the zone width remained trapped on the top of a vibrating packing. In this case, the packing becomes separated into two distinct fractions, whereas for segregation that is driven by particulate reorganizations, a vibrated bed develops a continuous gradation of particle sizes.

Thus there are at least two distinct mechanisms that are responsible for particle size segregation in vibrated granular materials. For practical applications, such as processing food or pharmaceutical powders, it is important to know whether convection or particle reorganization will dominate for any particular shaking process. Conventionally, in experiments on shaken powders, the acceleration of the base is used as the control parameter for a vibrating bed; but although this is a good measure for the motion of a single particle, it is unsuitable for the characterization of a disordered many-body system. The details of how, and where, free volume is introduced into a close-packed assembly of grains during 'shaking' are reflected in the granular dynamics and, hence, in the segregation behaviour. Clearly the segregation depends on many factors including the shaking amplitude (which affects the free volume that is introduced during expansion) and on the size and frequency of the forces transmitted to individual grains (not simply a question of the applied forces but dependent on particulate parameters such as friction).

The picture that emerges is that the convection mechanism¹ dominates for low-amplitude, high-frequency vibrations. But shake the system harder, and particle reorganizations² take over; the convection rolls become unstable, and the main mechanism for size segregation is then the competition between independent-particle and collective reorganizations. The competing domains of amplitude and frequency need to be investigated experimentally in order to find better control parameters for vibrating beds. This, with further theoretical work, may finally give a convincing answer to the question of which mechanism causes the Brazil nuts to rise. □

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A question of endosomes

Regis B. Kelly

NEUROTRANSMITTER-storing synaptic vesicles have only one function—to dock with the nerve-terminal plasma membrane and to fuse with it when the nerve terminal is electrically stimulated. Because they are so single-minded, analysis of their membrane proteins has revealed a great deal about how membrane docking and fusion occur^{1,2}. On page 537 of this issue³, Feany and Buckley unexpectedly bring actin into the exocytosis picture by showing that one of the main synaptic vesicle proteins, synaptotagmin, influences the actin-based cytoskeleton.

Besides encouraging new ideas about docking and fusion, this and two other papers^{4,5} give us insight into how synaptic vesicle proteins may be targeted to vesi-

cles. Neurons, and some non-neuronal cells, seem to have a specialized endosome in addition to the one that carries out housekeeping duties. In neurons, synaptic vesicle proteins are targeted to these specialized endosomes.

Fusion of synaptic vesicles with the nerve terminal's plasma membrane is triggered by an increase in cytoplasmic calcium. Among the synaptic vesicle proteins currently characterized, synaptotagmin is attracting the greatest attention because it binds calcium. Synaptotagmin has two of the C2 domains that allow a protein to move from a cytoplasmic to a bound form when calcium levels are raised⁶. Peptides corresponding to the C2 domains and antibodies against them inhibit exocytosis

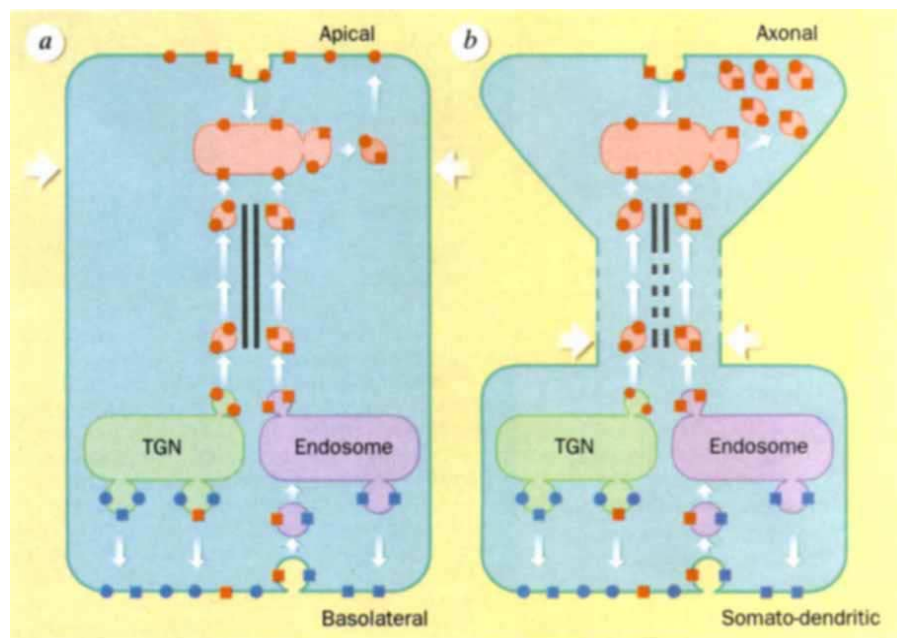


FIG. 1 Parallels between protein sorting in epithelial cells and neurons. *a*, In epithelial cells, newly synthesized proteins of the basolateral membranes (blue) go directly from the trans golgi network (TGN) to the basolateral membrane. Proteins (blue squares) that also have an endosomal targeting signal, such as the transferrin receptor, go to basolateral endosomes. Newly synthesized proteins of the apical membrane (red) have two options. They may go directly (red circles) to the apical membrane or they may go first to the basolateral membrane (red squares) from which they are taken into the basolateral endosome. This class of apical membrane protein is sorted into a transcytotic vesicle, which is transported to the apical surface. Both direct and indirect modes of transport to the apical surface require microtubules. It is not yet known whether the apical transport vesicles fuse first with the apical endosomes, as shown, or with the apical plasma membrane. *b*, Postulated membrane protein sorting in neurons. Proteins such as the transferrin receptor (blue squares) recycle through the endosomes of the cell body and dendrites, but do not enter the axon. Synaptic vesicle proteins such as synaptophysin (red squares) go to the cell body endosomes, where they colocalize with the transferrin receptor (blue squares)⁵. Brefeldin A, which disrupts transcytosis of membrane proteins in epithelial cells, induces the formation of tubules containing transferrin receptors and synaptophysin⁵. The other synaptic vesicle membrane proteins (red circles), synaptotagmin, SV2 and so on take the direct route and so are unaffected by brefeldin A. The two classes of membrane protein are transported by microtubules to the nerve terminal, where synaptic vesicle biogenesis occurs. In transfected fibroblasts, synaptotagmin remains in the plasma membrane, presumably because it lacks the correct post-translational modification, or cannot form a complex with other synaptic vesicle proteins. Arrowheads indicate barriers to membrane protein diffusion.

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in the neuroendocrine cell PC12 and in the synapse of the squid stellate ganglion^{7,8}. Mutants in synaptotagmin have markedly impaired neuromuscular transmission in *Drosophila*⁹ and *Caenorhabditis elegans*¹⁰. The evidence that synaptotagmin is a calcium sensor that regulates exocytosis begins to seem overwhelming.

The case is not closed, however. Calcium entering the nerve terminal regulates exocytosis but can also affect both the cytoskeleton that holds synaptic vesicles near their release site and the rate of endocytosis. Synaptotagmin could act as a calcium sensor regulating any of the three processes. Support for an alternative role for synaptotagmin comes from the observation of calcium-dependent exocytosis from neuroendocrine PC12 cells that completely lacked synaptotagmin¹¹. Nor is synaptotagmin the only potential calcium sensor. Another such regulator has recently been discovered, rabphilin, the receptor for the synaptic vesicle peripheral membrane protein, rab3A. Rabphilin also binds calcium and has a C2 domain¹².

Finally, we have the unexpected data from Feany and Buckley³ showing that synaptotagmin can cause filopodial extension in transfected fibroblasts. These observations are reminiscent of the induction of microvilli in transfected cells by the actin-binding protein villin¹³. Synaptotagmin does not seem to interact with actin directly, however, because double immunofluorescence images frequently show that it is excluded from actin-rich regions of the cell membrane (K. Buckley, personal communication). Perhaps competition between synaptotagmin and protein kinase C for a common binding site¹⁴ is crucial. Whatever the explanation of the unexpected morphological change, we are now compelled to consider more seriously the possibility that cytoskeleton rearrangements regulated by synaptotagmin occur during secretion.

When the synaptic vesicle protein, synaptophysin, was expressed in fibroblasts, it was targeted to endosomes, which was satisfying because synaptic vesicles arise by endocytosis. This simple picture has been distorted, however, by the reports that synaptotagmin remains in the plasma membrane³, and SV2 in an unusual, 80-nm vesicle⁴, in transfected fibroblasts. Exposure of neurons to brefeldin A tells us that, even in neurons, synaptophysin and synaptotagmin go to

different endocytotic compartments⁵. To make sense of the targeting data, we have to remember that there is more than one endosomal pathway in neurons. The endosomes in the neuronal cell body are devoted to housekeeping functions such as recycling low-density-lipoprotein and transferrin receptors. Endosomes in the

observations on the distribution of synaptic vesicle proteins in neurons⁵ and in transfected fibroblasts⁴. Different cell-surface proteins have different bio-synthetic routes in epithelial cells (Fig. 1a).

If this model is applied to neurons (Fig 1b), then a newly synthesized synaptic vesicle protein may travel from the plasma membrane via the cell body, housekeeping endosome to the apical, specialized endosome. When expressed in fibroblasts, each synaptic vesicle protein could arrest at a different step on the pathway, depending on what nerve-cell-specific information it needs to continue on its way.

This explanation requires that even fibroblasts have small vesicles derived from a specialized endosome to which SV2 can be targeted. How widespread are specialized endosomes? Epithelial cells can generate, by endocytosis from their apical membranes, small vesicles and tubules containing, for example, water, chloride and proton transporters. The vesicles, clustering under the apical membrane, make up a storage pool of membrane transporters that can be inserted when the cell is hormonally stimulated. A similar situation may exist in fat and muscle cells, which contain the glucose transporter, GLUT 4, and analogues of the synaptic vesicle proteins VAMP (ref. 15) and rab3A (ref. 16).

The presence of small vesicles in nerve, fat, muscle and epithelial cells, derived from a specialized endosomal population and mobilized to the cell surface by hormone stimulation, suggests that many if not all cells might have such a pathway. Its function would be to allow rapid but transient modification of the cell surface (Fig. 2). The data of Feany *et al.*⁴ are consistent with the presence of such a pathway in fibroblasts. □

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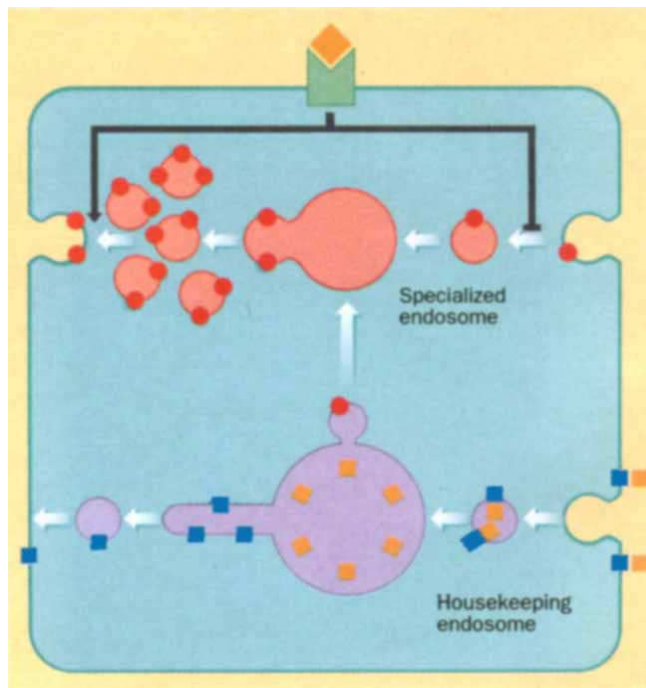


FIG. 2 The cell-surface modification pathway in cells. As discussed in the text, synaptic vesicle biogenesis can be viewed as an example of a pathway also present in epithelial cells, fat cells and muscle cells, where it is used to modify the cell surface. All cells have housekeeping endosomes, which are used to internalize low-density-lipoprotein receptors (blue squares), for example, and separate them from their ligands (yellow squares). The receptors recycle back to the cell surface via a bulk flow process¹⁷. Other proteins are sorted out into a specialized endosomal pathway that gives rise to a population of small storage vesicles. Fusion of these vesicles can be stimulated, and their endocytosis sometimes inhibited, when a hormone or neurotransmitter (yellow diamond) binds to a receptor on the surface of a cell. In this scheme, the synaptic vesicle is a modified storage vesicle.

axon and nerve terminals exclude such proteins. Synaptic vesicle proteins are targeted to axonal endosomes, so their biogenesis involves these specialized endosomes, not the housekeeping endosomes of the cell body.

The rules that govern the targeting of membrane proteins to the apical domain of epithelial cells also apply to axonal membrane proteins. So we can use the information on epithelial cells to interpret

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Towards phase III trials for candidate vaccines

Gordon Ada

ARE effective vaccines against the human immunodeficiency virus (HIV) close at hand, some way off or, as some have thought¹, unlikely to materialize? In *The Lancet*, David Schwartz and colleagues² report some encouraging findings with a candidate vaccine in phase I trials to assess safety and immunogenicity. They show that progress is being made. A phase I trial involves volunteers living an HIV risk-free life. A phase II trial is an extension of phase I, but also may include some (uninfected) participants whose lifestyle is not risk-free.

Schwartz *et al.* studied the immune response in humans to a preparation of the envelope protein of HIV (rgp120/HIV-1, strain IIIB, Genentech), produced in DNA-transfected Chinese hamster ovary cells and adsorbed onto alum, the only adjuvant currently licensed for general medical use. The reasons for using a subunit of the virus as the basis of an HIV vaccine is the perceived overwhelming requirement for safety. Despite the high efficacy of many live attenuated viral vaccines, the requirement for product safety, especially in the case of retroviruses, has favoured the subunit approach to the extent that all of the current candidate preparations in clinical prophylactic trials are of this type (see table), being mainly the envelope protein or part thereof. Two potential disadvantages of this approach are that the isolated viral protein may be poorly immunogenic (so that a series of injections with adjuvant are required); and that it might not contain sufficient B- and T-cell epitopes to overcome genetic polymorphism in human populations (so that only a proportion of immunized people produce protective responses).

The findings of the new trial are encouraging for three reasons. First, after three doses of either 100 or 300 µg, all recipients made antibody to the protein. Even more importantly, out of ten recipients of the higher dose, nine made antibody which neutralized homologous viral infectivity, and all made antibody which bound to a V3 domain peptide (the site of the principal neutralizing determinant) and which blocked binding to CD4 (the main cellular receptor for HIV). That is, there was a consistent response by the participants in the trial.

Second, total antibody titres after the second and third injections remained higher over time compared to some earlier results, though there is room for improvement. Third, in about half of the recipients of the higher vaccine dose, antibody was

detected which neutralized an antigenically distinct isolate (HIV-1_{SE2}). Cross-reactivity has also been observed following prolonged immunization with a similar preparation in studies in baboons³, and is generally attributed to antibody directed to strongly conformational but rather poorly immunogenic epitopes near the CD4 binding site^{2,3}.

A glycoprotein synthesized in DNA-transfected mammalian cells should mimic the properties of the native viral antigen, including glycosylation pattern and overall conformation. This favours its persistence, as antigen-antibody complexes on the plasma membrane of follicular dendritic cells in lymphoid tissues⁴, leading to longer-term production of antibody of increasingly higher affinity. For the initiation of T-cell responses, the

antigen, once inside an antigen-presenting cell, should be readily degradable to peptides that can bind to class II MHC molecules to form a complex recognized by the T-cell receptor. There was strong T-cell proliferation after the third dose of antigen, and it seemed to increase with time. But the observed substantial variation between individual responses means that the incorporation of additional T-cell determinant(s) would probably be useful. Some improvement in this respect, together with a broadening of the cross-reactive antibody response, might be achieved by priming with antigen of one specificity and boosting with antigen of a related specificity, an approach planned by the authors².

What of other options? Use of more potent adjuvants than alum could yield both a higher and broader cross-protective antibody response, and also induce class I MHC-restricted, CD8⁺ cytotoxic T-cell responses which could eliminate virus-infected host cells shortly after exposure of the host to virus. Given earlier findings (refs 5 and 6; see table), a

Candidate HIV-1 vaccines in clinical trials in uninfected individuals		
Candidate vaccine	HIV strain/cell type	Developer
Recombinant proteins		
rgp160	LAI/baculovirus LAI/vaccinia-derived MN/vaccinia-derived MN/LAI/CHO	MicroGeneSys ImmunoAG 160 Pasteur-Merieux, Connaught
rgp120	LAI/CHO IIIB/CHO MN/CHO SF2/yeast SF2/CHO	Genentech Biocine
Recombinant poxviruses		
Vaccinia-gp160	LAI	Bristol-Myers, Squibb
Canarypox-gp160	MN	Pasteur-Merieux, Connaught
Peptides		
MAPS-V3-Octomers	MN	United Biomedical
V3 loop unconjugated	MN	Pasteur-Merieux, Connaught
HGP 30	LAI	Viral Technologies
Ty, p24, VLP	LAI/yeast	British Biotech
V3 loop-purified protein derivative	MN	Swiss Serum and Vaccine Institute
Combinations		
Vaccinia-gp160 + five envelope products	IIIB, MN, SF2	Various companies
Vaccinia-gp160 + gp160 + three envelope peptides	LAI	G. Beaud and colleagues
rgp, HIV envelope (env) glycoprotein made using recombinant DNA technology. Recombinant poxviruses, poxviruses containing DNA coding for HIV antigens. Baculovirus, an HIV antigen product of insect cells infected with recombinant baculovirus. Vaccinia-derived, an HIV antigen product of mammalian cells infected with recombinant vaccinia virus. CHO, Chinese hamster ovary cells. MAPS, multiple antigenic peptides. HGP 30, a peptide sequence of the HIV gag protein. Ty, p24, VLP, a segment of the HIV gag protein, assembled as virus-like particles. LAI, MN, IIIB, SF2, antigenically different HIV isolates. Compiled with the help of Pat Fast, Division of AIDS, NIAID, Washington DC.		

Acting on actin

THE brush border of the intestine is made up of finger-like protrusions, the microvilli, each supported by a rigid core of actin bundles. The protein that organizes the actin filaments in this manner is villin. E. Friedrich *et al.* (*J. Cell Sci.* **105**, 765–775; 1993) have pursued their observation that cultured cells transfected with villin develop microvilli on their surfaces by exposing the cells to cytochalasin to block actin filament ends. This causes the microvilli to vanish and short actin rods to materialize in the cytoplasm. Taking the cytochalasin away again leads to the reappearance of the microvilli. So it seems that short actin filaments sit on the membrane, waiting to nucleate the microvillus actin cores — another striking example of the multifarious morphological manifestations of the actin molecule.

Friend or foe?

GLOBAL warming and ozone depletion are hopelessly entangled in the minds of many government ministers, but given that ozone and its man-made foes the chlorofluorocarbons (CFCs) are also greenhouse gases, they may be excused. A particularly clear head will be needed if considering the policy implications of a report by C. Clerbaux *et al.* (*J. geophys. Res.* **98**, 10491–10497; 1993). Assessing ten of the hydrohalocarbons being developed as substitutes for the CFCs, the authors warn that although the effects on ozone are indeed less severe, three of the substitutes (known as HCFC22, HCFC142b and HFC125) would trap more infrared radiation, molecule for molecule, than the CFCs they are replacing. Industrial applications may require heavier use of hydrohalocarbons than of CFCs to maintain efficiency, and the problem is likely to linger — these molecules can remain in the atmosphere for decades.

Fishy business

A YEAR ago, J. M. Burkholder and colleagues reported the discovery of a 'phantom' dinoflagellate responsible, through secretion of a toxin, for the mass death of estuarine fish. E. J. Noga *et al.* now describe a similar case — a dinoflagellate is again involved, but this time it afflicted fish kept in aquaria (*Vet. Rec.* **133**, 96–97; 1993). The victims were tilapia being acclimatized to brackish water; when they died mysteriously, Noga *et al.* found a hitherto unknown dinoflagellate was present in the water in large numbers. Experiments with more hapless tilapia showed that the dinoflagellates were stimulated to bloom by the presence of living fish, and that the lethal agent was a filterable toxin. More of these cryptic killers no doubt await identification, but that will probably require more sensitive detection techniques.

greater immune response, including that of cytotoxic T cells, might also be achieved if the product was used to boost the immune response in humans already primed with a recombinant avipox virus vector such as canarypox (ref. 7; see table); such a vector should be safe, even in immunocompromised recipients.

Although much more remains to be achieved (including a formulation which would induce strong, persisting mucosal responses, especially in the female reproductive tract), the publication of these results is timely. Similar candidate vaccines are already in clinical trials in HIV-infected individuals and are scheduled to be used in trials on HIV-infected mothers and children starting this year⁸. Plans are also underway to initiate efficacy studies in uninfected groups that have a high risk of infection. The World Health Organization has identified sites for such trials in four developing countries.

As well as sponsoring most of the trials listed in the table, the Division of AIDS at

the National Institute of Allergy and Infectious Diseases, Washington, recently established a working group to promote the development and testing of suitable candidate vaccines in phase III efficacy trials. There have been doubts that suitable candidate vaccines would become available in time, but the results of the phase I trial of the Genentech product suggest that a product of this type will be one such candidate. □

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ASTRONOMY

A nearby protogalaxy?

S. van den Bergh

OUR neighbours are an elderly bunch — if, that is, we are talking about galaxies. The 27 known members of the Local Group of galaxies (a loose association about 2 megaparsecs across) have travelled far down the evolutionary pathway; all but one have transformed more than 90 per cent of their baryonic mass from gas to stars. To watch the early stages of galaxy formation, we must look further afield. Now it seems that a young protogalaxy — one that has not yet started to form massive stars — may have been found in the relatively nearby M81 group of galaxies, only 3–3.5 megaparsecs away.

Last year, using the 30-metre IRAM telescope, Brouillet, Henkel and Baudry¹ tentatively identified a small intergalactic cloud on the eastern outskirts of M81 that was emitting radiation characteristic of carbon monoxide. Together with colleagues², they have now filled in the missing optical data and can confidently assert that the molecular cloud and its environs show no sign of massive star formation.

Tracer

Why the importance of carbon monoxide? The expected life history of a galaxy is a progression from atomic hydrogen or heavier elements recycled from older stars, through denser condensations of molecular species, in particular H₂, to the formation of new stars. Carbon monoxide is an excellent tracer for molecular

hydrogen, which is otherwise difficult to detect.

Radio observations in the 21-cm line of neutral atomic hydrogen³ show that the CO cloud is embedded in one of two massive atomic hydrogen (H I) complexes in the outer part of M81. The gas complexes do not seem to be directly associated with the spiral arms of this galaxy. It seems likely that they have been formed as a result of recent tidal interactions between M81 (NGC3031), M82 (NGC3034) and NGC3077^{4–7}, a threefold tug which may have disrupted the disk of M82 (ref. 8). It has been estimated⁴ that a quarter of all the hydrogen in the M81 group at present lies in tail and tidal structures outside the optical radii of the main cluster galaxies.

Dwarf galaxies might, it is thought, form tidal tails such as these^{9–11}. Henkel *et al.*² propose that their CO cloud is a protogalaxy that represents the missing link between tidal H I arms and active star-forming regions, such as that lying at the tip of one of the tidal arms in the interacting galaxies NGC4038 and 4039 (the Antennae).

What might happen next? An infant dwarf galaxy formed from a tidal tail might differ from more ancient dwarfs in several important ways¹². First, tidal arms are not expected to entrain much dark matter (because it is too 'hot'; that is, has too large a velocity dispersion to be trapped) so that young dwarfs formed in this way should have low mass-to-light ratios.

Second, most dwarfs formed from tidal arms should be relatively rich in metals. Inspection of the Palomar Sky Survey shows that most tidal arms are found in systems in which at least one component is a giant, or supergiant, spiral. Gas drawn from such luminous spirals (and the dwarfs formed from it) should be metal-rich, giving us a signature by which to identify young dwarfs.

This aim could be thwarted if there are strong metallicity gradients in spirals, making the outer regions (from which tidal tails are drawn) more metal-deficient than their interiors. But recent work by Oey and Kennicutt¹³ is reassuring. The abundance of oxygen relative to hydrogen is observed to fall in the range $8.8 < 12 + \log_{10} (\text{O}/\text{H}) < 9.2$ in the outskirts of most early-type spirals and $12 + \log_{10} (\text{O}/\text{H}) > 8.5$ in the outer regions of most late-type giant spirals, so young dwarfs formed from the tidal arms of most spirals will indeed be relatively metal-rich.

Swallowed

Inspection of the luminosity and metallicity data for dwarf galaxies assembled by Skillman, Kennicutt and Hodge¹⁴ shows that at least 95 per cent of their dwarf galaxies are too metal-poor to have condensed recently from tidal arms. Perhaps the explanation for this paucity of dwarfs formed from tidal tails is that many have been swallowed up again by collision (or tidal friction) with one of their ancestors. This suggests that young dwarf galaxies are likely to constitute only a small fraction of the total dwarf population in the nearer reaches of the Universe, so Henkel and colleagues are lucky indeed to have found one. □

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Birds lost in the red

James L. Gould

THE prolonged controversy over whether animals can sense the Earth's magnetic field has mostly given way to an equally lively debate about how the field is detected in the first place. Certain bacteria¹, insects^{2,3}, birds^{4,5} and fish^{6,7} seem to use miniature magnetic compass needles made of magnetite, and biologically generated and localized magnetite crystals have been found in a variety of other magnetically sensitive species⁸. On the other hand, some marine elasmobranchs

other's field. But in atoms with unpaired electrons — that is, with vacancies in the outer orbital — there is a net field; the Fe^{2+} atoms of iron in magnetite, for instance, have six unpaired electrons, each with a net field. These unpaired electrons tend to align themselves like compass needles with the Earth's field, and so create a slightly stronger local field. This phenomenon, known as 'paramagnetism', is extremely weak: the disruptive influence of thermal energy is

about 10^6 times stronger. But when, as in magnetite crystals, atoms with net fields are positioned so that the individual fields can interact to become mutually aligned end to end, the crystal as a whole can have a net field so strong that it is permanent (immune to disruption by thermal energy at physiological temperatures).

Leask's proposal is based on the observation that when a paired electron is excited to a higher energy level by the absorption of a photon of light, it can (very rarely) reverse its spin and thus be trapped in the higher orbital; only a second (equally rare) reversal of spin on its part



Australian silveryeyes — restless subjects.

detect the Earth's field inductively by swimming through field lines⁹, having anticipated the work of Oersted, Ampère, Faraday and Henry by many millions of years.

In contrast to these intuitively straightforward mechanisms is M. J. M. Leask's surprising 'optical-pumping' hypothesis, which proposes that magnetic fields are detected by photopigments in the eye¹⁰. The report on page 525 of this issue¹¹ by Wiltshchko *et al.* now provides dramatic evidence for this mechanism in a species of migrating bird, while that by Able and Able¹² on page 523 shows how the magnetic-field sense is calibrated against celestial cues.

All magnetism arises from the motion of electrons; the electrons moving through wires in the coil of an electromagnet, for example, generate a magnetic field. Even in magnetite (lodestone), it is electron motion that produces the field, but in a different way: electrons have 'spin', and this spin induces a tiny, very local magnetic field. Paired electrons always exist with opposite spins, and thus cancel out each

(or by the other member of the pair still at the lower level) will permit it to return to its previous position. As a result, when illuminated at an appropriate wavelength, a vast array of pigments (like the billions of molecules of rhodopsin in the retina) will have a certain population of molecules with an added net magnetic field. Leask proposes that the enhanced paramagnetism induced by such spin flipping could be somehow used to detect the direction of a magnetic field.

There are three major problems with this optical-pumping hypothesis. First, some species can orient themselves magnetically under starlight¹³ or in complete darkness¹⁴. Second, even the most generous calculations indicate that optical pumping could account only for direction detection, even in full daylight; the many responses of animals to small magnetic field changes⁸ (apparently connected with a map sense or circadian calibration) could not be based on the Leask mechanism. Third, there is no obvious way that the exceedingly diffuse paramagnetic behaviour of rhodopsin could be coupled to

R. Wiltshchko

the nervous system, particularly considering that the rhodopsin molecules in most vertebrate eyes have little of the consistent alignment the model requires. (This is not to say that conventional paramagnetism is not potentially important to animals; superparamagnetic magnetite crystals — crystals too small for their mutually aligned internal fields to hold their own against the Earth's field — are a strong candidate for detecting tiny magnetic field variations in some animals¹⁵.)

If, as most of us had parsimoniously hoped, there was only one way that animals other than elasmobranchs detect magnetic fields, then Leask's hypothesis could be ignored. Alas, the animals we study, and the natural selection that has fashioned their sensory strategies, appear once again to be more subtle and complex than would have been entirely convenient.

What Wiltschko *et al.*¹¹ have done is place silvereyes (an Australian species of bird) in a state of migratory restlessness into cages under different wavelengths of light and observe whether their attempts to escape are aligned with the migratory direction of their free-living peers. The experiment provided no access to non-magnetic cues, and previous tests had shown that this dusk- and dawn-migrating species, like many others, uses the Earth's field to orient themselves under these conditions. When illuminated with white, blue or green light, the birds were fairly well oriented; but under red light — 633 nm, a wavelength not absorbed by rhodopsin — the birds were disoriented. This result is remarkably consistent with Leask's model, and may explain Wiltschko and Wiltschko's earlier report that young homing pigeons (an age class very dependent on cues gathered *en route* to a release site) are disoriented if transported in total darkness.

How convincing now is the case for visually modulated sensitivity to magnetic fields? Almost the only striking paper before now was the report by Phillips and Borland¹⁶ that magnetically based orientation in newts can be altered by the spectral composition of light. Their results could reasonably be overlooked as essentially inexplicable: only blue-green light abolished orientation; bluer wavelengths had no effect; green, yellow and orange light rotated the animals 90° left of controls; red light was not tried. In the wake of the paper by Wiltschko *et al.* the distressingly complex proposal Phillips and Borland offer for their results will have to be taken more seriously.

As for the larger view, we must keep in mind that although optical pumping now seems a real possibility as a magnetic direction sense in birds that migrate during the day and at twilight, things are different for the many nocturnally migrating birds, and other classes of vertebrates,

invertebrates and animals of any stripe with higher levels of sensitivity to the Earth's magnetic field. For them the Leask mechanism is useless, and magnetite-based systems still appear to be the best bet¹⁷.

Another critical issue for any sort of magnetic compass is how to calibrate it. The need for calibration arises from the inconvenient placement of the Earth's magnetic poles some 2,300 km from the geographic poles (on Prince of Wales Island in Canada at only 75° north latitude, in the case of the north magnetic pole). The discrepancy between the rotational poles, about which all celestial cues revolve, and the magnetic poles produces a declination error that can be quite large, particularly in parts of North America and Europe. To compound the problem, the exact locations of the magnetic poles wander at a measurable annual rate (in 1800, for instance, the declination in London was 24° west, whereas in 1600 it was 11° east); moreover, during the course of geological time the poles have occasionally undergone enormous and rapid redeployments.

In their new paper¹², Able and Able show unambiguously (and somewhat surprisingly) that when this calibration is restricted to daylight hours, migratory birds use the axis of rotation of the Sun-centred pattern of polarized light in the sky rather than the Sun itself as their guide to true north. (Nocturnal calibration, on the other hand, depends on the axis of rotation of the pattern of stars.) Again, as a matter of perspective, it is important to remember that the bird's magnetic compass is strictly a backup system; in the presence of celestial cues — Sun, polarized light or stars — migrants and local navigators (such as homing pigeons) will opt for the more reliable information in the sky overhead⁸. □

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Infra-green plants

AGRICULTURE is a heavy industry these days. Irrigation, artificial fertilizers, mechanized monoculture, programmed pesticide spraying, and now genetically engineered plant varieties, have all raised crop yields ever higher. Indeed, only increasingly intensive agribusiness can keep the world's population growing. The more people there are in the world, of course, the nastier it is for everybody, and the more disastrous the crash will be when it eventually does come; but let's not worry about that. Daedalus now presents the green revolution with its next technical fix.

Green plants, he notes, waste much of the daylight that hits them. Chlorophyll has an absorption peak in the blue, and another in the red, but is relatively reflecting in the ultraviolet and the green — which is why plants look green. Better spectral matching could double the rate of photosynthesis, and therefore the growth-rate, of our crop plants. The required technology already exists, in those lurid posters whose colours seem brighter than daylight, and in the tinted markers for highlighting sections of a document. They contain a fluorescent pigment which absorbs light at one wavelength (typically ultraviolet) and reradiates it as a visible colour. So DREADCO's chemists are formulating a fluorescent crop-spray. It will absorb the wasted ultraviolet and green bands in daylight, and reradiate them as blue and orange light which the plant can use.

DREADCO's 'Fluorogro'® could simply coat the leaves like a highlight-marker. But Daedalus wants it to enter the leaf and bind intimately to the chloroplasts themselves, broadening their photochemical absorption like the sensitizing dye in film. A perfectly absorbing plant will look black — not a pretty sight for sentimental nature lovers. But it will grow twice as fast.

In cloudy regions like northern Europe and Siberia, Fluorogro will invigorate the crops like extra sunlight. And wherever the thinning ozone layer lets in extra solar ultraviolet, Fluorogro will seize this potentially damaging radiation and harness it for useful photosynthesis. The resulting spurt of crop growth will absorb the rising CO₂ content of the atmosphere, countering the greenhouse effect. Thus two ecological disasters will cancel each other out, while doubling the world's farm output. For the suburban market, Daedalus is also working on the converse strategy. DREADCO's 'Fluoroslo'® will shift the solar spectrum well away from the chlorophyll absorption bands. Sprayed on a verdant lawn, it will choke off photosynthesis and minimize the need for mowing.

David Jones

Nose stars and brain stripes

SIR — Mammals with sensory receptor specializations often have corresponding modifications in the brain that help us understand general principles of brain organization. A conspicuous and unique specialization is the star of the star-nosed mole's face, where 11 fleshy rays branch out from each side of the nose (*a* in the figure). Each ray is covered with domes

thetized star-nosed moles (*Condylura cristata*) by making small openings in the skull and recording the responses of neurons with microelectrodes. Sites where neurons responded vigorously to light touch on specific rays were marked with small electrolytic lesions. The anaesthetized moles were perfused with saline and fixatives, and cerebral cortex removed, flattened, frozen

and cut parallel to the surface into 60- μ m sections. Brain sections were processed for cytochrome oxidase, which enhances the barrels of somatosensory cortex of rats and mice³. In all processed hemispheres, 11 dark stripes of cytochrome oxidase-dense tissue separated by narrow septa of cytochrome oxidase-light tissue were apparent in S1. Most of the pattern appeared in single favourable sections from the flattened cortex (*b* in the figure), and the full pattern could be reconstructed from several adjoining sections. Because the number of stripes in S1 of each hemisphere conforms to the number of rays on each side of the face, and marking microlesions related most of the stripes to specific rays, we propose that each stripe represents the sensory input from a given ray in an isomorphic pattern.

The stripes of S1 of the star-nosed mole constitute a second dramatic example of how somatosensory cortex can be divided into distinct morphological structures, each devoted to a specific body part. The other well-known example is the dot-like barrel field of S1 in rats, mice and some marsupials²⁻⁴, where cytochrome oxidase-dense barrels of cells represent the vibrissae of the upper face. We believe that these patterns are explicit expressions of a widespread tendency for sensory inputs differing in neural activity patterns to be segregated in the brain⁵. Hints of morphologically distinct septa between representations of major body parts are apparent in S1 of some primates, but such

delimiting septa may be less apparent in larger-brained mammals, where neurons are less densely packed and peripheral specializations less pronounced.

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Cytokine-antigen vaccines

SIR — We read with interest the report of Tao and Levy¹ on the effectiveness of an idiotypic/GMCSF fusion protein as a vaccine. We have produced a similar antigen-cytokine vaccine, using less elegant techniques but nevertheless with success. Interferon- γ is an effective adjuvant when administered simply mixed with an antigen^{2,3}, but when we biotinylated it, and used avidin as an antigen, the cytokine-antigen conjugates were more immunogenic than simple mixtures or conjugates with inactivated cytokine. This success led us to suggest the potential of cytokine-antigen fusion proteins as vaccines⁴. It appears that, at least in our case, the cytokine-antigen vaccine was more effective than the mixture not because of improved contact between antigen-presenting cells and the cytokine (as higher doses of the cytokine did not have the same effect) but perhaps because the cytokine was focused on cells presenting the antigen rather than on others presenting irrelevant antigens.

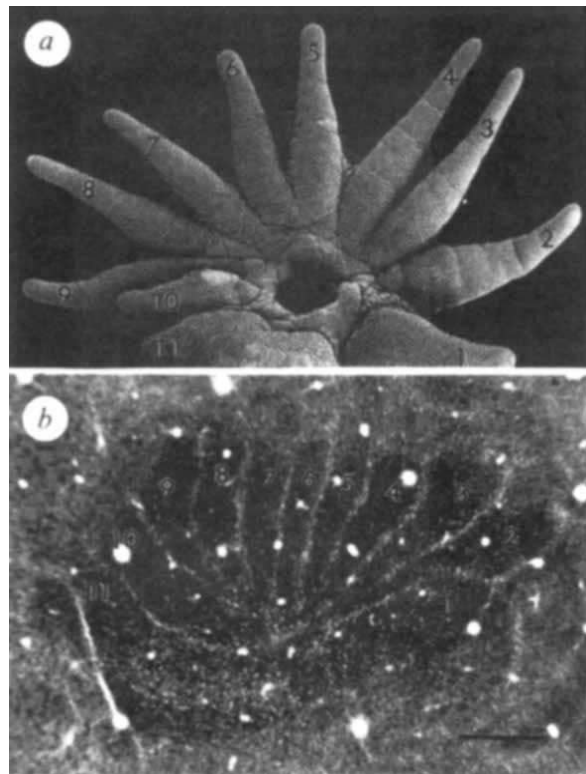
Whatever the mechanism involved, it seems that vaccines may need to contain elements to direct them to the appropriate antigen-presenting cell, and other elements to enhance antigen presentation by those cells, and/or to push the immune system in the required direction, for example to Th1 rather than Th2 responses^{5,6}.

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a, A rotated face-on view of the right half of the numbered fleshy rays of the nose surrounding the nostril. Up is right. *b*, An example of a brain section cut parallel to the surface of the somatosensory cortex and processed for cytochrome oxidase. The pattern of numbered ray-like stripes of darkly stained tissue is isomorphic with the rays of the contralateral half of the nose. Scale bar, 0.5 mm. Medial, up; posterior, right.

that cap underlying arrays of receptors and nerve endings called Eimer's organs¹. The rays are in constant motion and are repeatedly brought into contact with the substrate as the mole searches for food, thus seeming to be an important source of information about the external world. We expected this strange modification of the tactile surface of the nose to be reflected in the organization of the primary somatosensory cortex (S1), much as the sensory inputs from the whiskers of the face of rats and mice are expressed in a dot-like pattern of cortical 'barrels'². However, we expected the morphological units, if apparent, to be shaped like rays rather than hair follicles.

To test our assumptions, we identified parts of the somatosensory cortex responding to stimulating rays in five anaes-

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Hammerhead shark origins

SIR — Knowledge of macroevolutionary sequences provides insight into the origin of biological innovation and the nature of selective forces responsible for the evolution of adaptations. Hammerhead sharks (Family Sphyrnidae) are characterized by the presence of a remarkable innovation: a laterally expanded head (termed the cephalofoil) in which there has been substantial reorientation of olfactory, optic and electric field sense organs. Because head shapes across contemporary species differ markedly in the extent of lateral elaboration, knowledge of phylogenetic relationships among species allows estimation of the trajectory of cephalofoil evolution.

Previous phylogenetic analysis of morphology ordered taxa according to the absolute degree of cephalofoil lateral expansion; namely, the species with the least laterally expanded head is

regarded as ancestral, the most derived lineage is characterized by the most exaggerated cephalofoil, and species with intermediately expanded heads are ordered so that increasingly derived taxa have increasingly expanded heads¹. This hypothesis is intuitively appealing because it suggests that there has been directional selection for lateral expansion ('improvement'), and that the variations present in contemporary species are snapshots of stages in the progress of cephalofoil elaboration. To test this hypothesis, I determined 921 base pairs from two mitochondrial protein-coding genes for 8 taxa of hammerheads (7 species, 2 sub-species) across the full array of morphologies and for two outgroups.

Phylogenetic analysis of the DNA data provided support for previous claims that hammerheads constitute a monophyletic group, implying a single origin of the cephalofoil (see figure). However, the mitochondrial DNA phylogeny suggests a strikingly different sequence of hammerhead evolution than previously thought (see figure). Most notably, narrow-headedness (characteristic of *Sphyrna tiburo*) is derived, not ancestral. Furthermore, the lineage characterized by extreme head width (*Eusphyrna blochii*) is most ancestral. These data provide no information about the origin of the cephalofoil, and they argue that the variation in extant taxa provides misleading information about the trajectory of morphological evolution either because of the tendency to align things in progressive series or the assumption of parsimony. Instead, the data suggest that once the cephalofoil had originated, head width (relative to body length) underwent exaggerated expansion (along the *Eusphyrna* lineage), diminution (along the *S. tiburo* lineage), and remained unchanged at different times in hammerhead shark evolution.

The cephalofoil is thought to function as a bowplane to increase lift when the shark moves through the water and to enhance manoeuvrability when the shark twists, elevates or depresses its head. In addition, expansion of the head probably enhanced orientation and prey detection capabilities that would accompany increased number and/or separation of electric field, olfactory and ocular sense organs. Identification of at least two distinct functions provides the opportunity for selection to act in different directions, a possibility that may explain the existence of multiple cephalofoil designs. Descriptions

of ontogenetic and morphological variation within and among species and observations of the comparative performance of sharks whose heads are of different shape should explain the presence of different cephalofoil designs.

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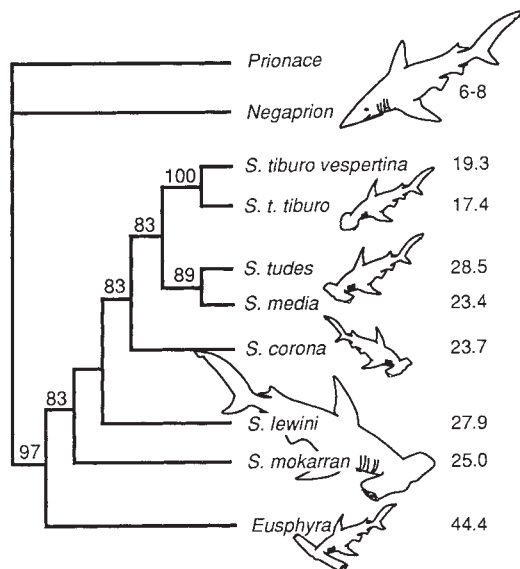
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Estimating extinction rates

SIR — Continuing patterns of human population growth and associated destruction of natural habitats lead many to believe that we are probably entering a period of mass extinction comparable in degree to, and swifter in time than, the great spasms of extinction in the geological past^{1,2}. The commonly cited estimates of extinction rates are, however, very rough; they derive mainly from species-area relations combined with projected rates of destruction of natural areas^{2,3}. Here, we use data compiled by the World Conservation Monitoring Centre (WCMC) to make some different, but still very rough, assessments of impending extinction rates.

Specifically, these data include documented animal and plant extinctions since 1600 (ref. 4); 'Red Lists' of animal species threatened with extinction, compiled every two years by the WCMC using the International Union for the Conservation of Nature's (IUCN) categories 'probably extinct', 'endangered' (survival unlikely if causal factors continue) and 'vulnerable' (likely to become 'endangered' if current trends continue), along with 'rare' (but not necessarily threatened), 'status unknown' and 'not threatened' (refs 5-7); and the WCMC database on seed-bearing plant species threatened with extinction, which uses comparable categories of threat.

Table 1 summarizes these data, showing the proportions of major plant and animal taxa that have become extinct since 1600 or are currently threatened. Although crude, these figures are illuminating, even though they mainly show the enormous variations in the attention different taxa have received⁸. Even for comparatively well-studied groups like birds and mammals, the numbers are thought to be serious underestimates because many tropical species have not received the



Phylogenetic hypothesis of relationships among species of *Sphyrna* and *Eusphyrna* based on DNA sequence data. Phylogeny was inferred using the branch-and-bound algorithm of PAUP (ref. 2) and using the neighbour-joining cluster algorithm on the matrix of corrected pairwise differences (implemented using PHYLIP; ref. 3). The neighbour-joining tree was identical to one of the two minimum-length topologies. This phylogenetic hypothesis is 35 steps shorter than the progressive evolution hypothesis¹, a difference that is significant based on likelihood analysis³. Numbers along the branches are the percentage of times that that branch was found in 250 bootstrap replications. Only values greater than 50 are shown. Cartoons of sharks provide an indication of the relative size and head shape of the species. Average head widths (expressed as a percentage of body length) are given for each species (from ref. 4). Data are for sharks that are 60–70 cm in length. Protocols for sequencing shark DNA, the DNA data and additional details of methods of phylogenetic inference are available from the author on request.

TABLE 1 Species in major taxa that have become extinct since 1600 or are threatened with extinction

		No. of species certified extinct since 1600	No. of species listed as threatened	Approximate total of recorded, extant species	Percentage extinct	Percentage threatened
Animals	Molluscs	191	354	10^5	0.2	0.4
	Crustaceans	4	126	4×10^3	0.01	3
	Insects	61	873	10^6	0.005	0.07
	Vertebrates	229	2,212	4.7×10^4	0.5	5
	Fishes	29	452	2.4×10^4	0.1	2
	Amphibians	2	59	3×10^3	0.07	2
	Reptiles	23	167	6×10^3	0.4	3
	Birds	116	1,029	9.5×10^3	1	11
	Mammals	59	505	4.5×10^3	1	11
	Total	485	3,565	1.4×10^6	0.04	0.3
Plants	Gymnosperms	2	242	758	0.3	32
	Dicotyledons	120	17,474	1.9×10^5	0.2	9
	Monocotyledons	462	4,421	5.2×10^4	0.2	9
	Palms	4	925	2820	0.1	33
	Total	584	22,137	2.4×10^5	0.3	9

TABLE 2 Dynamics of extinction and threatened extinction

Column		1 Threatened species joining lists (%)	2 Threatened species leaving lists (%)	3 No. of species becoming extinct	4 No. of species rediscovered extant	5 Characteristic extinction time (years)	6 Status level change (median)	7 Characteristic extinction time (years)
Animals	Molluscs	35	0.1	16	0	13,000	0.09	60,000
	Crustaceans	13	0.8	1	1	—	0.0	—
	Insects	10	4	1	1	—	-0.01	—
	Vertebrates	53	9	15	2	7,000	0.07	600
	Fishes	50	8	0	0	—	0.12	900
	Amphibians	31	0	0	0	—	0.03	3,000
	Reptiles	34	7	0	0	—	0.04	2,000
	Birds	62	14	14	2	1,600	0.05	350
	Mammals	46	1	1	0	9,000	0.07	250
	Total	39	7	33	4	—	0.05	15,000
Plants	Gymnosperms	0	0	0	0	—	0.0	—
	Dicotyledons	0.3	0.03	150	20	1,500	0.02	1,000
	Monocotyledons	0.1	0.1	13	5	6,500	0.01	1,700
	Palms	0.5	0	1	1	—	0.09	70
	Total	0.3	0.04	163	25	3,500	0.01	1,100

Time to 50 per cent extinction in column 5 is calculated from the net absolute extinction rate per year (column 3 minus column 4, divided by the number of years in the analysis). The median status level changes in column 6 are calculated by grouping the IUCN categories into the following levels: 'zero' (rare, status unknown or not threatened), V (vulnerable), E (endangered), Ex? (probably extinct), and Ex (extinct). The characteristic extinction time in column 7 is calculated from column 6 by determining the projected time to extinction of the threatened species in each taxon, if all those species are initially not threatened. Then, assuming that the species currently not on the lists will move as fast towards extinction as those on the lists, the minimum time to 50 per cent extinction is the ratio of half the total number of species to the number of threatened species multiplied by column 6.

careful attention needed for entry into the Red Data Books or for fully certified extinction². The numbers for insects are clearly nonsensical: for Britain's comparatively well-studied insect fauna, roughly 1 per cent has become extinct since 1900 and 7 per cent is listed as endangered or vulnerable, which is more like the world's birds and mammals in Table 1 and contrasts markedly with its 0.07 per cent of insects listed as threatened^{9,10}.

We can get an extremely rough idea of changing rates of extinction and endangerment by looking at the changes in the Red Lists for animals in 1986, 1988 and 1990, and in the WCMC Plants Database between 1990 and 1992. Table 2 shows that the animal Red Lists have undergone net growth of more than 30 per cent between 1986 and 1990. The status of threatened plants has, however, been less intensively studied at the global level than many animal groups, and the apparent

lack of growth of the plants database says much more about the rate of data entry than actual status changes. From 1986 to 1990 only 15 vertebrate species (and only 33 animal species in total) satisfied the stringent criteria for inclusion in the list of recorded extinctions. If this rate of extinction were accurate and sustained, it would be about 7,000 years before half the 45,000 or so recorded vertebrate species were exterminated. Similarly, from 1990 to 1992, an additional 163 plant species were recorded extinct in the plants database. Around 3,000 years would be required to extinguish half of all higher plants if this rate were accurate and sustained. These times are significantly longer than those deduced from species-area relations but they are very fast on an evolutionary timescale.

We can make a better — although still very crude — guess at how extinction rates might currently be increasing by estimat-

ing how fast the status of species already included in the lists is changing. In Table 2, any species whose status has changed during the time-frame of the analysis is given a positive or negative value according to the size and direction of the change (for example a change from being classed as 'vulnerable' to being 'probably extinct' would produce a value of +2). Column 6 in Table 2 shows the median status changes for some major taxa. The last column gives estimated times to extinction, obtained by assuming all species are not initially threatened, and dividing the number of status changes required for extinction (+4) by the median status change per species per year. For insects and other poorly-studied groups (including most plants), these numbers again tell us more about lack of information and rate of data entry than about extinction. But for higher vertebrates and the comparatively well-studied palms they possibly

give more useful numbers. For birds and mammals, this line of analysis implies extinction of half the species within roughly 200–300 years, and for palms it suggests 50–100 years.

We re-emphasize that the data in the Red Lists have been compiled opportunistically rather than systematically, so that these projected rates of extinction probably owe more to the vagaries of sampling effort and data entry than to real changes in the status of species. However, we believe that the approach does have potential merit in suggesting a new line of investigation of the dynamics of species extinction. The consequent estimates of extinction rates for the better-known taxa

are of the same order of magnitude as those derived from totally unrelated species–area relations, and they suggest that the time available for research into other plant and animal groups may be severely limited.

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Triplex model of chromosome ends

SIR — Sundquist and Klug¹ demonstrated that synthetic models of *Tetrahymena* chromosome telomeric terminus, consisting of the DNA duplex and the single-stranded 'tail' (T₂G₄)₂, stably dimerize in monovalent salts owing to quadruplex formation by the two tails. We have found that in physiological buffer containing about 100 mM Na⁺ and 10 mM Mg²⁺ the

also virtually fully protected. Some protection is visible also for the terminal block of the tail.

At 10 °C and in the presence of Mg²⁺, the molecules move in the gel as a single band with mobility corresponding to the mobility of monomers (data not shown), although we observed formation of dimers described by Sundquist and Klug¹ in experiments without magnesium after long incubation. For the incubation times we used in our experiments in Fig. 2, dimers never form (our unpublished observations and ref. 1). This means that the clear-cut protection

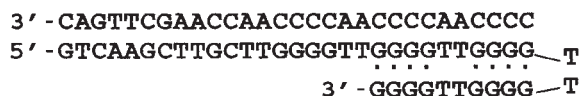


FIG. 1 Synthetic *Tetrahymena* chromosome terminus forming the proposed foldback triplex structure.

same model of chromosome ends forms an intramolecular pyrimidine–purine–purine triplex: the single-stranded G-rich overhang folds back, lying into the major groove of the terminal duplex forming the CGG base triads (Fig. 1). This new structure of telomeres provides a possible explanation for the stability of the ends of chromosomes *in vivo* against digestion and recombination.

Figure 2 presents our data on probing of the structure with dimethyl sulphate (DMS). Without Mg²⁺, we observe homogeneous modification of guanines along the whole G-rich strand (lanes 1–3). In the presence of Mg²⁺, a completely different pattern of modification is observed: one can see the clear-cut protection of guanines in two blocks within the duplex adjacent to single-stranded tails (lanes 4–6). Moreover, at least one of two guanine blocks belonging to the tail is

effect against methylation in Fig. 2 results from an internal structural rearrangement in the molecule. We therefore assume that in the presence of Mg²⁺ ions the single-stranded tail folds back, forming a pyrimidine–purine–purine triplex consisting of the CGG base triads (Fig. 1).

The model in Fig. 1 explains all major features of the results in Fig. 2 and is fully consistent with the available data on the CGG type of pyrimidine–purine–purine triplexes^{2–5}. Formation of the pyrimidine–purine–purine triplex leads to the complete protection of guanines in the duplex against modification by DMS^{4,5} and, as we have recently shown, formation of the CGG base triad leads to significant protection of guanines in the third strand⁴.

Our foldback triplex model provides a natural explanation for the stabilizing effect of chromosome ends. Occupation of the major groove and concomitant

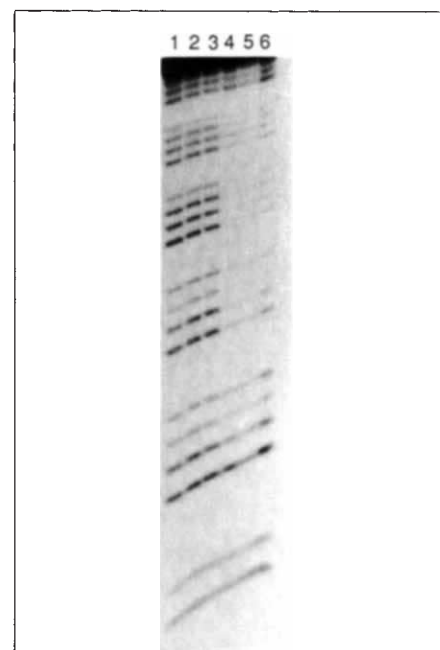


FIG. 2 Modification of the molecule in Fig. 1 by DMS. Lanes 1–3, 20 mM Tris–HCl pH 7.5, 1 mM EDTA plus 50 mM NaCl, 100 mM NaCl and 150 mM NaCl, respectively. Lanes 4–6, 20 mM Tris–HCl pH 7.5, 10 mM MgCl₂ plus 50 mM NaCl, 100 mM NaCl and 150 mM NaCl, respectively. The duplex was modified with 0.5% DMS for 25 s at 20 °C in the appropriate buffer. Alkylated DNA was cleaved in 10% hot piperidine and the product analysed in denaturing 15% polyacrylamide gel.

deformation of the minor groove due to triplex formation at the very end of chromosomal DNA should dramatically decrease vulnerability of the ends of chromosomes as targets for enzymatic cleavage. One can also assume that the foldback triplex structure would protect the telomeric ends against digestion by both 5'–3' and 3'–5' exonucleases. It is also quite reasonable to assume that the structure in Fig. 1 would prevent the telomeric ends from participating in recombination events.

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The good, bad and ugly

Nicholas Wade

Bad Science: The Short Life and Weird Times of Cold Fusion. By Gary Taubes. Random House: 1993. Pp. 503. \$25.

THE scientific method is a device to compel objectivity and restrain the natural urge of experimenters to believe in their own ideas. One of the more surprising lessons of the cold fusion episode is how many researchers pay scientific method scant attention when it gets in their way. *Bad Science* relates how not only the protagonists of cold fusion but many scientists who claimed confirmatory results were willing to neglect such basic cautions as running controls, understanding their experimental apparatus and checking their results before publication.

When Stanley Pons and Martin Fleischmann announced at a press conference on 23 March 1989 that they had initiated nuclear fusion at room temperatures, their claim had not been accepted by any peer-reviewed journal. Within a few weeks it also became clear that they had failed to conduct proper controls and had no credible data to prove that their experiment produced neutrons, tritium or even excess heat. What they had was nothing. Yet they started an avalanche that swept up scientists all over the world, mesmerized gullible backers from the Utah state legislature to the Electric Power Research Institute, and wasted tens of millions of dollars of seemingly not-so-scarce research funds. Even now the rubble has not completely ceased to jitter.

The main scientific elements of this bizarre tale have already been well told, for example by Frank Close in his book *Too Hot to Handle* (Allen Lane/Princeton University Press, 1990; reviewed in *Nature* 350, 29 (1991)). But Taubes, through extensive reporting, has added a wealth of detail that makes the episode a case study in the sociology of science, as well as an often hilarious chronicle of the extremities of human folly.

Pons and Fleischmann refused to be interviewed, so their side of the story could not be given here. *Bad Science* also recounts the antics and errors of many others who got drawn into the quicksand of cold fusion. Electrochemists at Texas A&M University gave cold fusion a major lift when they reported finding excess heat from cells with heavy water and platinum electrodes. But they, like Pons and Fleischmann, had neglected to do controls with ordinary water. Only after their press conference, when they did the controls and found that those too produced excess heat, did they realize that they were looking at an experimental artefact; the excess heat was caused by a thermometer

that was acting as a second cathode.

Scientists at the Georgia Tech Research Institute held a press conference to announce that they had generated neutrons from a Pons–Fleischmann cell. Later, when they became more familiar with their neutron counters, they learned that the devices were so sensitive to temperature as to respond to the warmth of a human hand as if to a burst of neutrons.

A committee appointed by the US Department of Energy made the rounds of all laboratories claiming to have detected cold fusion. One of its members, Allen Bard, returned from these visits deeply depressed by the low quality of science he had seen. The proponents of cold fusion paid no attention to details such as measurement errors or statistical fluctuations. "It was as if, when confronted with salvation they could ignore common sense, as if nature wouldn't possibly lie to them on such an important matter", Taubes comments.

The author is deliciously comprehensive in listing those who lent their support in varying degrees to cold fusion. The editorial page of *The Wall Street Journal* attributed criticism of cold fusion to "the compulsive naysaying of the current national mood". The *Salt Lake City Tribune* regularly boosted the home team's discovery. More surprisingly, so did *Newsweek* with a rash endorsement that "As Pons and Fleischmann found, sometimes the long shots do pay off."

Where newspapers led, sober scientific societies followed. To let cold fusion proponents present their results at an early meeting, the American Chemical Society waived its by-law prohibiting presentation of results that had not been peer reviewed. The by-law's purpose had been to discourage dissemination of specious findings. Favouring the positive, the Electrochemical Society arranged a symposium calling for "confirmation results" only.

Another clear sign of ir-

rational forces at work was that, following the egregious example of Pons and Fleischmann, the many chemists who jumped aboard the fusion train were surprisingly insouciant, indeed almost completely negligent, about protecting themselves from the intense radiation that their experiments should have generated if successful. Taubes notes an apocryphal joke that went the rounds, on how to tell the difference between a chemist and a physicist doing a cold fusion experiment: "The chemist believes fusion is occurring and shields the apparatus with a plastic dishpan. The physicist does not believe fusion is occurring, and shields the apparatus with tons of lead bricks."

Taubes has an agreeably sardonic style in perfect keeping with the ripeness of his material. *Bad Science* furnishes many serious lessons for the historian. Just as physiologists learn about the body's normal functions from its pathology, young researchers might usefully study this book, since it bears compelling witness to the human mind's irrepressible propensity for self-delusion. □

Nicholas Wade is science editor of *The New York Times*.



MARASMIUS oreades, the 'fairy ring' fungus. The picture was painted in 1894 by Beatrix Potter, today perhaps better known for her creation of *The Tale of Peter Rabbit*. Hundreds of her natural history watercolour drawings, mainly of fungi, can be seen in the Armitage Trust collection in Ambleside, England, and are reproduced in *A Victorian Naturalist: Beatrix Potter's Drawings from the Armitage Collection* by E. Jay, M. Noble and A. S. Hobbs. Warne (Penguin), £25.

Usage and abuse

John Allen Paulos

200% of Nothing: An Eye-Opening Tour Through the Twists and Turns of Math Abuse and Innumeracy. By A. K. Dewdney. Wiley: 1993. Pp. 192. \$19.95, £12.95.

A. K. DEWDNEY's new book is a pleasant listing of instances of innumeracy taken from the media, advertising, government, gambling and personal finance. It begins and ends with discussion of a few basic arithmetic and probabilistic ideas, percentages, ratios, expected values and the like, but its heart is in its examples. The former "Mathematical Recreations" columnist for *Scientific American* states that he was apprised of these abuses by "math detectives" from across North America.

Consider the first of these, which suggests the book's title: a light-bulb advertised to save 200 per cent of one's energy bill. Dewdney considers possible derivations of this figure, including those involving bizarre physical principles, and decides that it may have come about from dividing the energy saved by the energy "unsaved". If the energy saved is two-thirds, the 200 per cent figure results. He goes on in the first chapter to remark on the difficulty of quickly estimating deaths from natural disasters, the perils of informal risk assessment and the tendency we all have to filter our enquiries, making only those observations that confirm our beliefs. Broken into two-page snippets, the book moves quickly from one misuse to another.

Discussing misleading diagrams and graphs, what he calls "chart abuse", Dewdney gives contemporary illustrations of all the standard failings — different units, flawed perspectives, unlabelled axes, and the confusion of areas and volumes with linear measures. There is a nice section on sampling variability, especially among mutual fund managers whose differential successes can be quite adequately accounted for by chance. Dewdney also deconstructs claims such as "Life jackets are six times as safe", supports a magazine columnist's analysis of the best strategy to follow on a popular game show, suggests that prices with 9s in them should be rounded up, and considers the problem of false positives in medical and drug testing. Brief treatments of compound growth, scientific notation and the importance of informal estimation occasionally give way to more general remarks and exhortations.

The mathematical infelicities noted are sometimes unintended, sometimes deliberately misleading. The former is cer-

tainly the case with the computer company whose advertisements boasted that it had the highest price-performance ratio in the industry. Conscious deception, on the other hand, was probably the motive of the pocket-dictionary publisher who claimed that the dictionary contained 90,000 words, but neglected to mention that they were used to provide the 8,000 definitions.

The issue of baseless precision is broached in the context of a camera advertisement featuring a rare bird whose scientific characteristics are listed, in particular its average weight of 226.8 grams. The correspondent who brought this example to Dewdney's attention wondered at the remarkable exactness of this number until he realized that it was merely a literal translation from another system of measurement: 226.8 grams is simply half a pound. Dewdney, who gives idiosyncratic names to these various solecisms, terms the unwarranted digits "dramadigits".

Innumeracy is a perennial problem and any attempt to alleviate it is to be applauded. My primary complaint about this otherwise fine, right-thinking compendium is that it is not clear for whom it is written. Knowledgeable sorts, such as readers of *Nature*, will be amused by some of the examples but are unlikely to find much that is new. And neither they nor the people to whom the abuses cited are novel will be seduced by the book's writing or organization which, though serviceable, are not particularly engaging. Despite the cute terms scattered about ("dimensional dementia", "num", "percentage pumping"), the book's tone tends to be a bit bland. On a 0–10 scale, I'd give it a 7.231548. As the numerate reader knows, however, a book review is merely a sample of size one. □

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Principles of modern volcanology

Lionel Wilson

Volcanoes: A Planetary Perspective. By Peter Francis. Oxford University Press: 1993. Pp. 443. £50, \$98 (hbk); £25, \$49.95 (pbk).

THE phrase 'planetary exploration' probably conjures thoughts of the many spacecraft that have examined the surfaces and atmospheres of most of the bodies in our Solar System during the past two decades. But the same period has seen great advances in understanding the broad aspects of the evolution of our own planet.

Not least of these has been the new view of the Earth produced by the use of sonar-related techniques to map and obtain images of the floors of the oceans. These new data have fundamentally changed our perception of geological processes. In particular, we now realize that volcanism has been the primary process for the generation of almost all the rocks, and a great proportion of the topographic features, of planetary surfaces. Volcanology is also an excellent example of an area of Earth science in which important theoretical advances can readily be seen to



In the renowned *Campi Phlegraei*, with its hand-coloured engravings, Sir William Hamilton gave admirable descriptions of the eruptions of Vesuvius from 1766 to 1794. These examples appear in *Volcanoes: Fire from the Earth* by Maurice Krafft, who died with his wife Katia in the eruption of Mount Unzen, Japan, in 1991. The book is the latest in Thames and Hudson's New Horizons series of compact paperbacks, which impressively combine hundreds of illustrations with concise and informative texts.

have been driven by the need to understand the geological surface features of other bodies in the Solar System.

In his latest survey of volcanology, Peter Francis approaches the subject with these broad concepts very much in mind. Nevertheless, the work is organized around the styles of volcanism found on the Earth. This is not surprising, and indeed is probably inevitable, for the Earth has a wider variety of volcanic activity than anywhere else in the Solar System. This is simply because of the great variety of environmental conditions into which molten rock emerges at the surface of the Earth. On the deep ocean floors, melts encounter water at high pressures (tens of megapascals). On land, magma erupts into the much lower atmospheric pressure, commonly allowing gas exsolution and explosive activity. These eruptions take place through dry rocks in arid areas, through permafrost at high latitudes and through water-saturated rocks in most other places. Not even Mars, the most Earth-like of the other planets, could provide quite such a great variety of environments, even in its volcanic heyday, and conditions are even more restricted on planetary bodies that either are (Venus and Io) or have been (our Moon) volcanically active. But these bodies aid our understanding of volcanic processes by providing a wide range of values of the quantities controlling the style of eruptive activity, such as gravity, surface temperature and atmospheric pressure, and all of these issues are addressed by Francis.

The work cannot quite be classed as a popular account, for it includes a lot of the detailed physical, chemical and mathematical background required for a quantitative appreciation of how and why volcanic processes occur. But the lay reader is skilfully guided around or over the technical hurdles without the storyline being lost; and perseverance, when it is needed, is rewarded by many fascinating details about particular eruptions. Equally, the book does not truly qualify as a formal textbook: the list of references, mainly to papers in journals, at the end of each chapter is not always as exhaustive as would be needed by anyone hoping to arrive at an up-to-the-minute understanding of the field. These comments are not meant to be too critical, however, for the thoroughness and range of the coverage in the text make this an excellent adjunct to the reading list for even a postgraduate course in volcanology. Overall, Francis has succeeded in producing an extremely readable, entertaining, authoritative and informative work that should bring a better appreciation of modern volcanology to a wide audience. □

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Power and paper

A. Rupert Hall

Francis Bacon: History, Politics and Science, 1561–1626. By B. H. G. Wormald. *Cambridge University Press*: 1993. Pp. 409. £45, \$69.95.

THE image of Francis Bacon, Lord Verulam, as the ‘father of experimental science’, well established as it is, has been much challenged (or rather, broadened) in recent books. Everyone knows that Bacon met his death from a cold caught by stuffing a chicken with snow to preserve it; less familiar is his assertion: “I care little about the mechanical arts themselves; only about those things which they contribute to the equipment of philosophy”. The “bringing down as from heaven a shower of inventions at once useful and new” was an inducement to the unfolding of this philosophy, not an end in itself. Bacon admired the historians and moralists of antiquity, as he did some of the moderns — Machiavelli (in the *Discourses* on Livy), Philippe de Commines, Guicciardini; both ancient and modern natural histories he regarded as defective, and he had neither understanding of nor regard for the mathematical sciences. His interest was always in the general rather than in the particular: “let no man look for much progress in the natural sciences — especially in the practical part of them,” he wrote, “unless natural philosophy be carried on and applied to [the] particular sciences and particular sciences be carried back again to natural philosophy.” For lack of this philosophical nourishment, the particular sciences had hitherto stagnated.

True, Bacon as a good lawyer believed in the supreme importance of well-attested facts or “instances”. Sound progress in knowledge would be achieved not by following the “intellect left to itself” but by “resting constantly on experience and advancing step by step”. It was essential to gather systematically such “instances”, from which generalizations (“axioms”) might be “educated by a certain order and rule” which “shall in their turn

point out the way again to new particulars . . . For our road does not lie on a level, but ascends and descends first ascending to axioms, then descending to works” (*Novum Organum*, I, Aph. 103). While Bacon’s logic of natural science has been much examined, less attention has been paid to its context and generality, for Bacon anticipated that the same procedure would pay dividends in the social and political sciences also. “It is plainly the case”, we are assured by Wormald, “that Bacon both in offices of power and also on paper — two exercises which he did not separate — attempted



Francis Bacon — flawed intellectual giant whose interest was “always in the general rather than in the particular”.

civil science.” It should not be surprising — though it is notorious — that Bacon himself, with common law and statecraft as his profession and with human history and the light it casts upon man’s moral nature as his best learning, should have advanced little even in natural history, the only field he tackled. If by his method and example in science Bacon (in Wormald’s words) “helped

men in later years", it was in matters of state that "his confidence and vision proved correct even in particulars".

Wormald, surely the ablest of our elder historians, here firmly sets Bacon in his epoch as the budding statesman of Elizabeth I's time bidding to inherit the Cecil legacy under James I. For Bacon, a humanist and a man of religion, the truths of divinity were confirmed by history and experience; and although Truth was (he declared) the daughter of time not of authority, time had for him only validated divine authority. Yet although history was judged by Bacon to be the least exceptionable branch of human knowledge, it is impossible, Wormald affirms, that it "should have stood higher in Bacon's reckoning than philosophy". Philosophy permitted the highest exercise of human reason, subject to the provisos that "all knowledge is limited by religion, and to be referred to use and action". (Pure mathematics referred to neither.)

The Bacon of court and cabinet studied here was no godfather to the Royal Society or to industrial science, nor was it his logical "machine" (Bacon's word) that transformed seventeenth-century science. Wormald discloses not a philosopher in the modern sense but a politician, scholar and moralist who indeed wrote science like the Lord Chancellor that Bacon became. The author points out that the logical machine was never tested — for the natural history to which it must be applied was never assembled — and so its defects, already seen by such close successors of Bacon as Marin Mersenne, were never revealed.

Wormald's portrait, ranging as it does over the whole of a life and a vast mental range, is not drawn in strong lines but in delicate shading. His prose is often as elliptical as his subject's, and many sentences must be read twice. Like Bacon's road to true knowledge, his way ascends and descends through Bacon's multifarious writings. His book has no obvious structure, chronological or other, and is often (deliberately) repetitive. He cheerfully flouts the canons of 'contextual' history and does not hesitate to leap forward in his comments to the nineteenth and twentieth centuries. To my mind, this rich, complex study repays the effort that must be expended on it: to make Bacon tidy is to distort him. To fail to relate his *Essays* to the *Novum Organum* is to risk incomprehension. Bacon's extant writings are so large, his exposition so elusive — perhaps befitting a contemporary of the metaphysical poets — and his topics are so varied that debate about them will not soon cease; Wormald shows what a vast, and at the same time flawed, intellectual giant Bacon was. □

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Ways of seeing

Patricia S. Goldman-Rakic

A Vision of the Brain. By Semir Zeki. Blackwell Scientific: 1993. Pp. 366. £18.95, \$36.95.

THE primate visual system is usually considered the model example of integrative neurobiology and is the only 'systems' research to be acknowledged by a Nobel prize (to David Hubel and Torsten Wiesel in 1981). All students of neuroscience know about the pathways from retina to cerebral cortex, ocular dominance, orientation columns and cytochrome oxidase blobs in the primary visual cortex, as well as the developmental permutations of the numerous connections in the visual cortex and its physiological properties. These details are plainly exposed in every modern textbook of neurobiology and discussed in many review articles. It may be wondered whether a book on the visual system is necessary and what it could contribute beyond all this material.

Yet Semir Zeki's *A Vision of the Brain* is new and fresh because it is at once thematic, evaluative and didactic. The primary facts of visual cortex structure and function are reviewed with clarity and skill, and, for anyone who is unfamiliar with the visual system, the book will provide a highly readable, convenient, clear and up-to-date summary, though not of all components, nor all levels of analysis (such as the molecular biology or biophysics of colour-sensitive receptors in the retina). The central theme, expertly covered, is the cortical basis of colour vision. The clinical and experimental evidence for the existence of a specialized colour centre outside the primary visual cortex is explained, evaluated and discussed in an exemplary Socratic style. The history of ideas about visual specialization, viewed by someone who has been part of this history for the past 25 years, is especially interesting. Zeki's account of blind spots and revelations leading to our present highly sophisticated knowledge of the architecture of vision is enlightening. It will be of broad appeal and educational value to neuroscience graduate students, an invaluable introduction for non-neuroscientists, especially psychologists, computer scientists, historians of science and philosophers, and an encouragement to those scientists who find themselves 'swimming upstream'.

The science is strategically interwoven with historical and critical commentary. For example, in Chapter 10, Zeki captivates interest with the startling sentence: "In 1983, a miracle happened" to introduce Zeki's pivotal clinical case of motion blindness in a patient with all other aspects of visual processing otherwise pre-

served. Chapter 13 is a diversion that champions anatomy and physiology and may displease a few computational and computer scientists: "To become meaningful to the experimental neurobiologist, the computational neurobiologist needs the facts of the nervous system even more than the experimental neurobiologist needs the theory generated by the computational neurobiologist. . . Theory has always been of importance in the collection of facts, including even the mere anatomical facts." David Marr's much admired book *Vision* and the popular "what and where doctrine" are also discussed, critically and fairly.

Does the visual system provide a vision of the brain? It is obvious that as a prototype, it has instructed and inspired the study of many other areas of the cortex. But columns were first described in the somatosensory cortex, and functional specialization in the motor cortex and the language areas of the frontal and temporal lobes. As pointed out by Zeki, knowledge of other systems, such as the motor cortex, should have had more influence than they did on visual scientists, but important discoveries were ignored. Visual scientists tend to be myopic when it comes to knowledge beyond vision. The problems of binding and integration, for example, may not be solved within the visual system. Nevertheless, it is difficult to argue with the thesis that the operating principles of this system provide, particularly with respect to parallel processing, the key to the functional organization of the cortex as a whole.

The book is enjoyable to read, not only because of its intellectually appealing content but also because it is beautifully produced and well illustrated. Short chapters, each designed to make a specific point, allow it to be read in parts, put down and picked up again, as with a good novel. Most important, the book shows how powerful ideas can blind us as well as lead us to reality; it is about belief systems and how our stated or tacit theories colour our perception of facts, even in the 'objective' world of science. This is not new in itself but it is something we need to be reminded of from time to time. That is why the book is important reading for seasoned investigators, budding scientists and undergraduates. If it encourages scientists to acknowledge their blindspots and consider how what they know fits with the knowledge they have eschewed, it will have greatly widened the field of view. □

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■ New in paperback is *Vision: Coding and Efficiency* edited by Colin Blakemore (CUP, £22.95, \$34.95). Reviewed in *Nature* **351**, 24; 1991.

Muscle deficiency and neonatal death in mice with a targeted mutation in the *myogenin* gene

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Myogenin is a muscle-specific transcription factor that can induce myogenesis in a variety of cell types in tissue culture. To test myogenin's role *in vivo*, mice homozygous for a targeted mutation in the *myogenin* gene were generated. These mice survive fetal development but die immediately after birth and show a severe reduction of all skeletal muscle. Myogenin-mutant mice differ from mice carrying mutations in genes for the related myogenic factors Myf5 and MyoD, which have no muscle defects. Myogenin is therefore essential for the development of functional skeletal muscle.

MYOGENIN, MyoD, Myf5 and MRF4 are muscle-specific transcription factors that belong to the basic-helix-loop-helix (bHLH) class of DNA-binding proteins¹⁻⁴. On the basis of their activities in tissue culture, it was anticipated that these proteins would play a crucial part in the differentiation and maintenance of skeletal muscle in mammalian embryos¹⁻⁴. However, both *myf5* and *myoD* can be inactivated in mice by gene targeting with no apparent effect on muscle development, raising questions about the functions of these proteins *in vivo* and possible redundancy among them^{5,6}. Further uncertainty about the role of myogenic bHLH proteins *in vivo* arises from the observation that mutant mice lacking *myf5* exhibit a severe rib deficiency⁵.

The evidence for myogenin, MyoD, Myf5 and MRF4 involvement in myogenesis is based principally on gain-of-function experiments in cultured cells, where they can act as potent myogenic factors^{1,2}. Expression of any of the myogenic factors can convert a variety of tissue culture cells into muscle cells¹⁻⁴. Converted cells behave like myoblasts in their responses to growth factors, genes they express, and ability to fuse into multinucleated myotubes. Conversion is mediated by myogenic factor binding to regulatory regions of muscle-specific genes and the activation of their expression.

The four myogenic regulatory proteins share considerable amino-acid sequence similarity, particularly in a 70-amino-acid bHLH domain essential for protein oligomerization, DNA binding, and activation of myogenesis in cultured cells¹⁻⁴. The regulatory regions of many muscle-specific genes, including those of the myogenic bHLH factors themselves, contain the DNA consensus binding site for myogenic factors known as an E-box (CANNTG)¹⁻⁴. Thus, these proteins may regulate their own and one another's transcription. Unique protein sequences outside the bHLH domain are likely to contribute to specificity, suggesting that myogenic bHLH proteins may not be functionally identical¹⁻⁴.

Although much has been learned about the functions of myogenic bHLH proteins in tissue culture, relatively little is known of their functions during embryogenesis. Genes encoding the four myogenic factors are activated during embryogenesis in a temporally distinct pattern⁷⁻¹¹. Transcripts for Myf5 appear first in rostral somites of the mouse at 8 days post-coitum⁷. Myogenin is expressed next, with transcripts appearing in the myotome by day 8.5, followed two days later by MyoD transcripts and other markers of terminal differentiation^{8,9}. MRF4 is expressed transi-

ently in the myotome between days 9 and 12 and is then repressed until after birth^{10,11}. In developing limb buds, Myf5 is initially detected between days 10.5 and 11.0; myogenin and MyoD appear half a day later⁹. Whether this specific timing is important for muscle development is unknown, but it suggests that each factor may have a distinct role during development.

Gene knockout experiments have demonstrated that Myf5 and MyoD are not strictly required for muscle differentiation. Although similar analyses have not yet been described for myogenin or MRF4, myogenin has features suggesting it may be essential for myogenic differentiation^{8,12}. Myogenin is expressed in all skeletal muscle cell lines examined so far, whereas many skeletal muscle cell lines do not express MyoD, Myf5 or MRF4 (ref. 4). Furthermore, antisense oligonucleotides that inhibit myogenin expression prevent myoblast differentiation^{13,14}.

To test myogenin's role in embryonic muscle development directly, we generated mice homozygous for a targeted mutation in the *myogenin* gene. These mice survive fetal development but are born immobile and die immediately after birth. They have severe reduction of differentiated skeletal muscle throughout their bodies, despite normal levels of MyoD. These results are in striking contrast to those obtained with targeted mutations of *myf5* and *myoD*, in which there is no effect on muscle^{5,6}. Our results demonstrate that myogenin is an essential component of the regulatory pathway leading to skeletal muscle formation during embryogenesis.

Targeted mutation of the *myogenin* gene

The murine *myogenin* gene, located on chromosome 1 (ref. 15), contains three exons and two introns spanning 2.5 kilobases (kb) of DNA¹⁶. To target *myogenin* sequences in embryonic stem (ES) cells, we made a replacement vector containing an 8-kb genomic fragment that included the entire *myogenin* gene plus 5.5 kb of 5' and 3' non-coding DNA (Fig. 1a). A *neo* cassette was cloned into the first exon in reverse orientation 25 codons downstream of the initiator methionine codon¹⁷. A herpes simplex virus (HSV-1) thymidine kinase (tk) cassette was positioned adjacent to the short arm for negative selection¹⁷ (Fig. 1a). This vector was electroporated into ABI ES cells¹⁸, and G418-FIAU doubly resistant colonies were selected. Two separate ES cell subclones, identified as having been targeted, were used to generate germ-line chimaeric mice, which were subsequently bred to produce heterozygotes carrying the targeted

mutation. As described below, mice generated from either ES cell line showed the same phenotype (Table 1).

Male and female mice heterozygous for the targeted mutation grew and bred normally, indicating that loss of one functional *myogenin* allele had no overt effects. When heterozygotes were mated, normal-sized litters resulted but about a quarter of newborns were stillborn. Southern analysis of genomic DNA from sixteen litters showed all dead pups were homozygous for the targeted mutation, whereas surviving pups were either heterozygous or wild-type (Fig. 1b; Fig. 2a; Table 1). So far, we have found no live pups with a homozygous mutant phenotype.

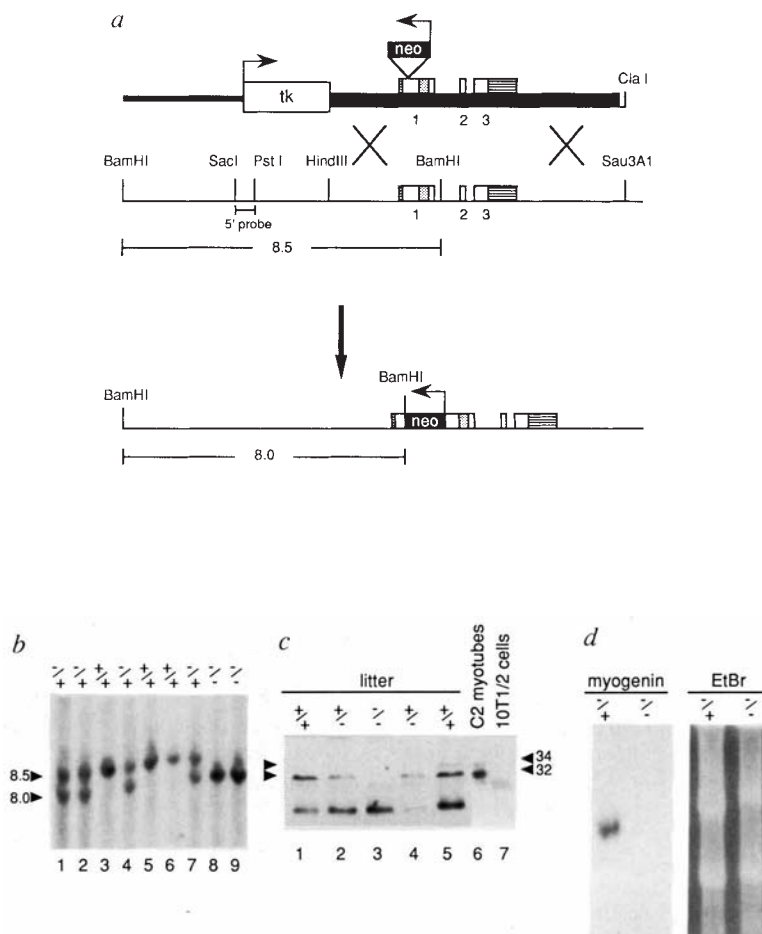
FIG. 1 Homologous recombination at the *myogenin* locus.

a, Schematic representation of the targeting event. The exons of the *myogenin* gene are numbered. The first exon contains the N-terminal activation domain and the bHLH region (indicated as a shaded box within exon 1) required for DNA binding and protein oligomerization²⁵. The second exon contains part of the C-terminal activation domain, and the third exon, the remainder of this activation domain, the translational termination codon, and the entire 3' untranslated region. Translated regions are indicated by unfilled boxes, with the bHLH region in exon 1 represented by a shaded box. Untranslated regions at the beginning and end of the gene are represented by boxes with horizontal lines. A unique *SacI* site was introduced into the first exon upstream of the bHLH region. An 8-kb *HindIII*-*Sau3A* fragment of this mutated clone was inserted into pMC1TK¹⁸, and the *SacI* site used for insertion of a *neo* cassette (derived from pMC1neopA; Stratagene) in the opposite orientation. The thickest line in the targeting vector represents the region homologous to the *myogenin* locus, the thinner line, vector sequences. Targeted events were detected by 'mini' Southern analysis²⁶ of *Bam*HI digested genomic DNA hybridized to a *SacI*-*Pst*I fragment located 5' to the homologous region in the vector. The targeted allele gave rise to an 8.0-kb fragment versus an 8.5-kb fragment with the wild-type gene. Out of thirty G418-FIAU resistant colonies examined, ten were found to be targeted. The negative selection using FIAU resulted in a 3.5-fold enrichment of targeted events. b, Genomic Southern analysis of a litter resulting from a heterozygous (*myg*^{ml}/+) cross showing that the targeted event in the *myogenin* gene correlates with the mutant phenotype. Pups 1-7 were surviving littermates; pups 8 and 9 exhibited the mutant phenotype and died at birth. c, Western analysis of myogenin-mutant embryos. A monoclonal antibody against the carboxy terminus of myogenin¹⁹ was used to probe for proteins extracted from 14.5-day embryos with the indicated genotypes. Extracts from two litters representing four +/+, ten *myg*^{ml}/+ and four *myg*^{ml}/*myg*^{ml} embryos were analysed for levels of myogenin protein and five examples from one litter are shown. Specific reaction of the antibody with myogenin was seen in differentiated C2 myocytes as two bands, corresponding to *M*_s 32 and 34K, as before²⁷. No specific reaction was seen in 10T1/2 fibroblasts, which do not produce myogenin. Bands migrating below the myogenin doublet in embryo lanes are nonspecific. d, Northern analysis of myogenin-mutant embryos. RNA (15 µg) from each sample was analysed for myogenin transcripts using a ³²P-labelled probe corresponding to the region of the *myogenin* gene 5' of the insertion site of the neomycin-resistance gene. The probe detects a single myogenin transcript of ~1.4 kb in RNA from heterozygotes, and no transcript from homozygous mutants. Ethidium bromide (EtBr) staining of RNA is shown to confirm equivalent loading.

METHODS. Homologous recombination at the *myogenin* locus was as follows: the replacement vector plasmid was linearized with *Cl*al and 10 µg electroporated per 10⁷ ES cells. Thirty FIAU-resistant (0.2 µM) and G418-resistant (180 µg ml⁻¹ active ingredient) ES cell clones were selected and analysed by Southern hybridization^{17,18,26}. As a result of homologous recombination, a diagnostic *Bam*HI site was introduced into the targeted allele so that a *Bam*HI digest of genomic DNA would differentiate between an 8.5-kb *Bam*HI wild-type fragment and an 8.0-kb mutated fragment identified using the *SacI*-*Pst*I fragment as probe. A second diagnostic genomic DNA digest with *Xba*I, producing an 11-

Hence the targeted mutation (referred to as *myg*^{ml}) is lethal in the homozygous state.

Western analysis using a monoclonal antibody against the carboxy terminus of myogenin¹⁹ and extracts from 14.5-day embryos showed that heterozygous mice produced about half the level of myogenin as wild-type littermates, suggesting that reducing myogenin levels by a factor of two does not noticeably affect muscle differentiation (Fig. 1c: lanes 1 and 5 versus 2 and 4). However, no myogenin protein was detected in homozygous mutants (Fig. 1c, lane 3). The western analysis is sufficiently sensitive to detect 3-5% of the wild-type amount of myogenin



kb fragment with wild-type DNA and a 12.5-kb fragment with mutant DNA, was hybridized with a probe representing a *Sph*I-*Xba*I fragment 3' to the vector homology region to confirm the presence of the mutated allele (not shown). An *Eco*RI digest, hybridized to myogenin cDNA, and polymerase chain reaction (PCR) were also used to confirm the presence of the mutant. To generate the heterozygous mice, two positive ES cell clones were injected into 3.5-day C57B6/6J blastocysts¹⁸. The relative contribution of ES cells in the resulting chimaeric and heterozygous mice was assessed by coat colour using the dominant agouti locus¹⁸. For each clone, three chimaeric males (all between 80-100% agouti) were bred to C57B6/6J females; 95-100% of the offspring from each male were agouti (111 animals). The diagnostic 8.0-kb *Bam*HI fragment was seen in 34 of the agouti offspring. For western analysis of myogenin, 14.5-day embryos were removed from the mother, homogenized in 0.4 M NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂ and protease inhibitors (PMSF, aprotinin, leupeptin and benzamidin, all at 0.5 mM), and insoluble material removed by centrifugation at 100,000g over a sucrose cushion. 25 µg total protein from each embryo and 25 µg nuclear extract from C2 and 10T1/2 cells were analysed by SDS-PAGE. Myogenin was detected using anti-myogenin monoclonal antibody¹⁹, horseradish peroxidase-conjugated goat anti-mouse IgG and ECL detection reagents (Amersham).

protein. Thus it is likely that the targeting event results in a null mutation at the myogenin locus and no myogenin protein is synthesized.

Using a myogenin complementary DNA probe to sequence upstream of the *neo* cassette, no myogenin transcripts were detected in the mutant mice by northern analysis (Fig. 1d). A myogenin cDNA probe containing sequences downstream of the insertion did identify transcripts, but these represent initiation within the neomycin cassette resulting in an untranslatable RNA (data not shown). Although it is conceivable that low levels of an N-terminal-truncated myogenin protein could accumulate in homozygous mutants, we believe this to be unlikely because the myogenin antibody is directed against an epitope near the C terminus and therefore could detect a truncated protein if it were expressed at levels as low as 3–5% of wild type. Additionally, as the heterozygous mice displayed no phenotype, a putative truncated myogenin is unlikely to play a dominant negative role.

Physical features of myogenin-mutant neonates

The *myg^{ml}/myg^{ml}* mice were phenotypically identifiable at birth as they appeared cyanotic and were immobile. Morphologically, they displayed abnormal curvature of the spine (kyphosis), thickening in the dorsal neck area, and less muscular limbs than their live littermates (Fig. 2a). The *myg^{ml}/myg^{ml}* pups weighed 15–20% less than their surviving siblings. At birth the mutant pups showed no movement and were unresponsive when prodded. Mutants were found to be alive at birth when we dissected them and observed their hearts beating; examination of their lungs showed that they had not inhaled.

Gross anatomical analysis of the skeletal muscle in dissected *myg^{ml}/myg^{ml}* pups revealed dramatic abnormalities compared with wild-type or heterozygous animals. Muscle tissue was greatly reduced throughout the body. The diaphragm was extremely thin and skeletal muscle throughout was consistently reduced in mass compared to non-mutant siblings. Multilocular adipose tissue (brown fat) was more extensive in the mutant neonates, particularly in the dorsal cervical region where it caused the observed thickening on the dorsal side of the neck (Fig. 2a).

Skeletons prepared from *myg^{ml}/myg^{ml}* pups revealed additional defects: most notably, an abnormal curvature of the vertebral column and a deformed rib cage (Fig. 2b). Ribs of mutant mice were abnormally curved, about 15% shorter (dorsal to ventral) than controls, and were abnormally aligned perpendicularly to the vertebral and sternebrae bodies. The mutant sterna were only 55% of the average length of controls (Fig. 2b, c) and were excessively ossified, with intersternebrae cartilage either reduced or absent^{20,21}. The first pair of ribs joined the manubrium nor-

TABLE 1 Genotypes of neonates from heterozygous (*myg^{ml}/+*) crosses

ES cell clone	+/+	Number of progeny <i>myg^{ml}/+</i>	<i>myg^{ml}/myg^{ml}</i>
13	13	21	5
26	17	32	4

Numbers represent three mating pairs producing seven litters from ES cell clone 13 and three mating pairs producing nine litters from clone 26. The nine *myg^{ml}/myg^{ml}* mice all died at birth; the 30 wild-type and 53 heterozygous mice survived. Many pups born dead were found too late to be genotyped or were destroyed by their mothers before they could be analysed, resulting in a lower than expected value for the *myg^{ml}/myg^{ml}* genotype.

mally, but the second pair joined late or not at all, resulting in excessive ossification and reduction of intersternebrae cartilage. Intersternebrae cartilage was more prominent caudally (Fig. 2c) and the degree of sternal ossification varied among mutant individuals. In addition, the cranial-most sternebrae were wider and the caudal-most sternebrae were narrower than controls (Fig. 2c). Elsewhere, mutant and non-mutant skeletons showed comparable degrees of bone development and ossification, but the *myg^{ml}/myg^{ml}* skeletons were more fragile, possibly as a result of reduction of associated musculature.

Histology of neonates and embryos

Histological sections through limbs of wild-type and heterozygous littermates showed densely packed muscle fibres, whereas those of the homozygous mutants had reduced fibre density (Fig. 3a). The regions where limb muscle would normally be present were populated instead by mononucleate cells and only occasional myofibres (Fig. 3a). Although myofibre density was severely diminished in the homozygous mutants, the number of nuclei in the muscle-forming regions of these animals was approximately normal, suggesting that these cells did not continue to proliferate. Besides the limbs, reduced skeletal muscle was particularly noticeable in the mutant tongue and diaphragm (Fig. 3b, c). Trunk and facial muscles showed similar reductions in myofibre density (data not shown).

The cells that populated the regions of neonatal mutant mice where muscle would normally be present could, in principle, be myoblasts arrested in the differentiation pathway, differentiated myocytes unable to fuse, or non-myogenic cells. To distinguish among these possibilities, we stained these regions with an antibody against myosin heavy chain (Mhc). Mhc was expressed in the residual myofibres, but not in the surrounding cells of limbs

FIG. 2 *myg^{ml}/myg^{ml}* neonates display a mutant phenotype. a, Neonates that died immediately after birth were photographed and genotyped along with surviving littermates. Those that displayed a *myg^{ml}/myg^{ml}* genotype are shown at the top, those at the bottom displayed a wild-type (right) or heterozygous (left) genotype. b, Neonates were stained for cartilage and bone using alcian blue and alizarin red respectively²⁸ (*myg^{ml}/myg^{ml}* mouse, right; wild type, left). c, The sterna of a heterozygote (top) and a *myg^{ml}/myg^{ml}* neonate (bottom).

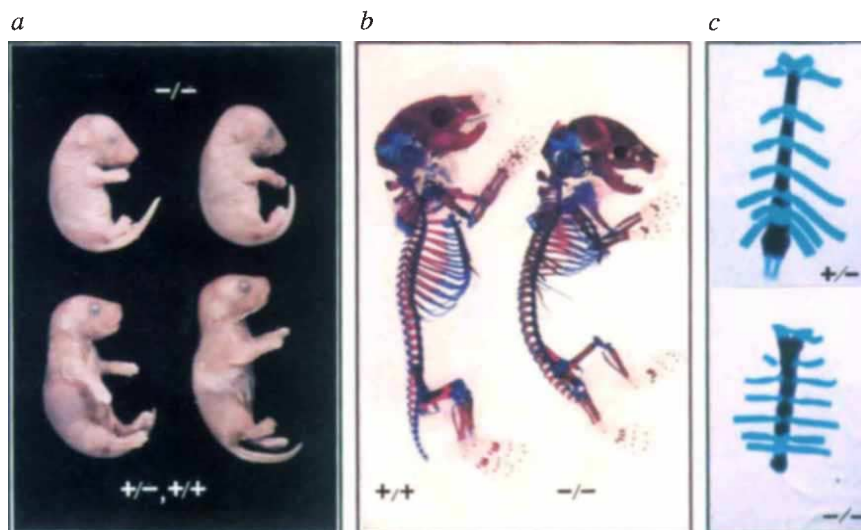
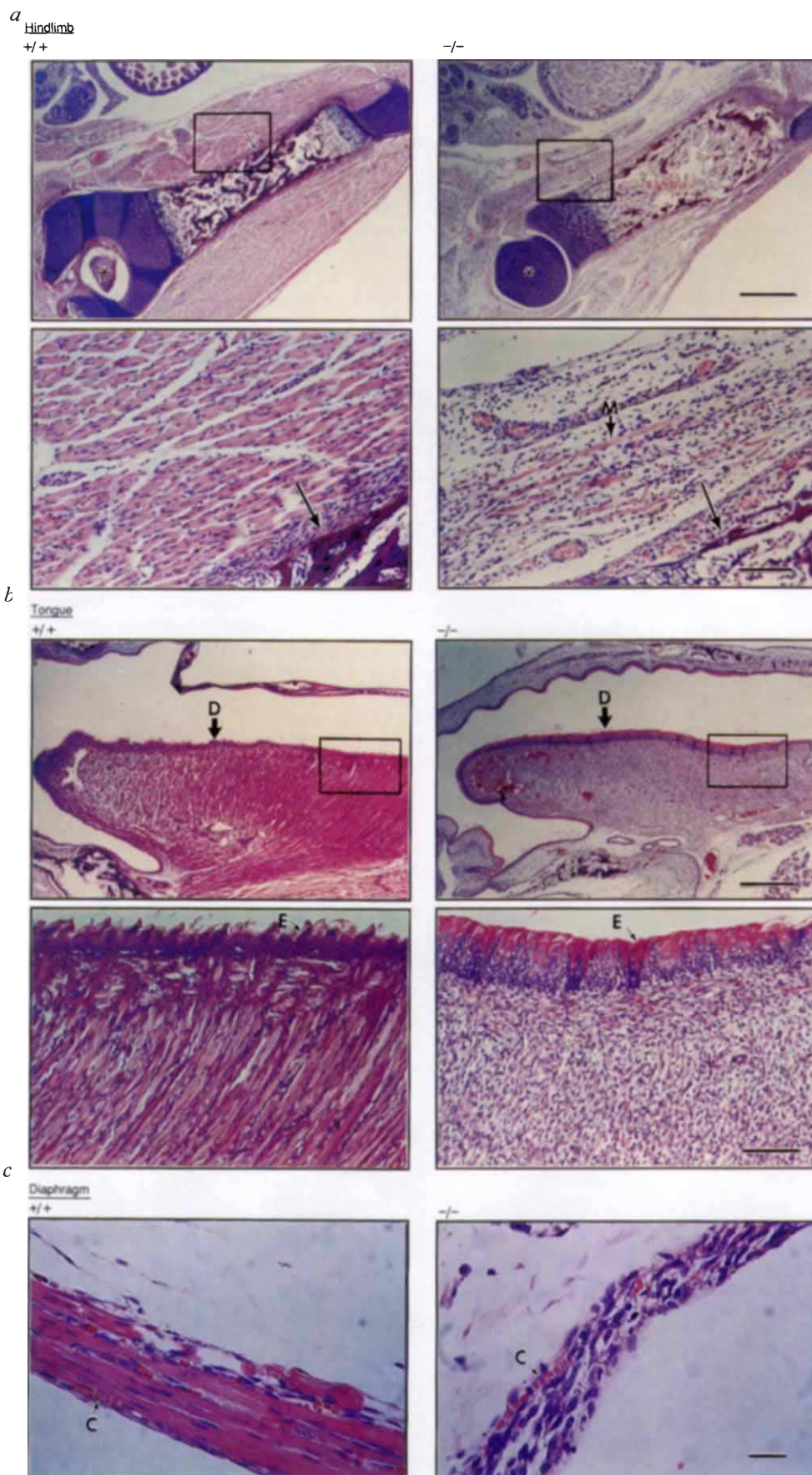


FIG. 3 Histological analysis of neonates carrying the *myg*^{mi} allele. Sagittal sections from three *myg*^{mi}/*myg*^{mi} mice (-/-) were compared to those obtained from three non-mutant siblings (+/+). *a*, Sections through the hindlimb and pelvic girdle of *myg*^{mi} mutant neonates revealed reduced numbers of muscle fibres (M) compared to those in the limbs of wild-type (+/+) littermates. Lower panels correspond to boxed areas in upper panels. Arrows indicate that the sections are approximately equivalent sections through the innominate bones of the pelvis, and asterisks indicate the head of the femur. *b*, Histological analysis of tongues from *myg*^{mi} mutant (-/-) and wild-type (+/+) mice. Lower panels correspond approximately to boxed areas in upper panels. Arrows indicate the dorsal surfaces of the tongues (D); 'E' indicates keratinized epithelium on the dorsal surface at higher magnification. *c*, Histological analysis of diaphragms from *myg*^{mi} mutant (-/-) and wild-type (+/+) mice. Sections are from the ventral portions of each diaphragm; 'C' denotes sections through a capillary. Scale bars in the upper panels of *a* and *b* represent 500 μ m; in the lower panels, 100 μ m; and in *c*, 20 μ m.

METHODS. Three *myg*^{mi}/*myg*^{mi} and three +/+ neonates were killed, skinned and then fixed for 24 h in 4% paraformaldehyde in PBS at room temperature, dehydrated through an ethanol series, placed in xylene and embedded in paraffin. Sections were cut 4- μ m thick and stained with haematoxylin/eosin.



of mutant neonates (Fig. 4a). Reduced skeletal muscle in the myogenin-null mice was also evident when assessed by staining of tongue sections with phalloidin, which labels filamentous actin (Fig. 4b). These experiments demonstrate the importance of myogenin for biochemical and morphological differentiation of skeletal muscle cells.

In an attempt to define further the unfused cells in the muscle-forming regions of the mutant mice, we examined MyoD protein expression by immunostaining. We were unable to detect MyoD protein in either the mutant or wild-type neonates (data not shown). This probably reflects the decline in MyoD expression that normally occurs before birth³. In day-15.5 embryos, however, MyoD was detected at comparable levels in nuclei throughout the muscle-forming regions of the limbs of wild-type and mutant embryos (Fig. 4c). Together these results suggest that myogenin is not required for commitment of cells to the myogenic lineage, but is important for terminal differentiation. The properties of the unfused cells that populate the regions of the neonates where muscle would normally be present still need to be defined.

Histological analysis of other tissues in mutant neonates revealed no gross abnormalities. Smooth muscle of the aorta and digestive tract appeared normal, and differentiated cardiocytes were observed in mutant hearts.

Muscle-specific transcripts in *myg^{ml}/myg^{ml}* mice

Myogenin activates a broad array of muscle-specific genes in cultured cells^{1,4}. To ask whether muscle-specific gene expression

was altered in the *myg^{ml}/myg^{ml}* mice, RNA from homozygous and heterozygous pups was analysed by northern blotting with muscle-specific probes (Fig. 5). As expected, there was a significant decrease in the transcripts for muscle creatine kinase (MCK), Mhc and the α - and γ -subunits of the acetylcholine receptor (AChR) in mutants. No difference was seen, however, in the transcript levels of the AChR δ -subunit (Fig. 5). As the α -subunit contains the ligand-binding site and all four subunits are required for proper receptor assembly, the number of functional cell-surface ACh receptors should be dramatically reduced in myogenin-mutant mice. These results show that absence of myogenin can affect the levels of many, but not all, muscle-specific transcripts.

As myogenic bHLH proteins crossactivate one another's expression in cultured cells, we investigated whether the absence of myogenin disrupted the expression of other members of this family. Transcripts encoding MyoD were present at normal levels, but those for MRF4 were decreased fourfold (Fig. 5). Normal MyoD transcript levels in myogenin-mutant mice indicate that myogenin is not required for the expression of the *myoD* gene *in vivo*. In contrast, myogenin may be necessary for normal levels of MRF4, although the cause of the decrease could be indirect owing to a decrease in the number of cells expressing MRF4. As there is so little differentiated muscle in the *myg^{ml}/myg^{ml}* mice, normal levels of MyoD messenger RNA suggest that MyoD is present in non-fused cells, but we have not been able to detect MyoD protein immunochemically in the mutant neonates. Regardless of where MyoD is expressed in the mutant

FIG. 4 Staining of muscle in wild-type and mutant neonates and embryos. a, Transverse frozen sections (10 μ m) through the hindlimbs of wild-type and mutant mice labelled with anti-Mhc. Muscle fibres (arrowed) in mutants were sparse and separated by cells that failed to stain with anti-myosin or other muscle markers. Scale bars, 50 μ m. b, Sagittal frozen sections (8 μ m) through tongues of neonates labelled with FITC-phalloidin, which stains filamentous actin. Muscle fibre density is reduced in mutants compared with non-mutant siblings (upper panels). Scale bars, 500 μ m. The tongues of wild-type neonates showed well differentiated fibre bundles that run longitudinally and transversely, whereas the tongues of mutants contained only short, sparse fibres which were most dense at the periphery (lower panels). Scale bars, 200 μ m. c, Parallel transverse frozen sections through day-15.5 non-mutant and mutant embryos labelled with anti-MyoD1. Levels of staining were comparable in both mutant and non-mutant embryos. Scale bar, 500 μ m. Arrows indicate the same regions at high and low magnification. Higher magnification reveals nuclear localization of anti-MyoD1. Scale bar, 50 μ m.

METHODS. Tissues and embryos were dissected and either frozen immediately in -65°C isopentane (limbs) or fixed in 4% paraformaldehyde in PBS overnight, rinsed in PBS and infiltrated with 0.5 M sucrose in PBS before freezing (tongues and day-15.5 embryos). For immunocytochemistry, sections from unfixed tissue were fixed in methanol at -20°C for 10 min and rinsed with PBS before processing. Sections were blocked with 5% normal goat serum in PBS (for 1 h at room temperature), incubated in a 1:20 dilution of polyclonal anti-Mhc (skeletal; Sigma) or 1:100 anti-MyoD1 for 1–16 h and then washed in PBS. Specific labelling was detected after incubating for 1–2 h in a 1:200 dilution of rhodamine-conjugated goat anti-rabbit antiserum (Cappel) followed by washing with PBS and mounting in 1:1 PBS:glycerol. To reveal filamentous actin, sections were fixed in 4% paraformaldehyde in PBS for 20 min and rinsed with PBS before incubating for 30 min in $5\text{ }\mu\text{g ml}^{-1}$ FITC-phalloidin (Sigma) in PBS. After staining, sections were rinsed in PBS and mounted as described.

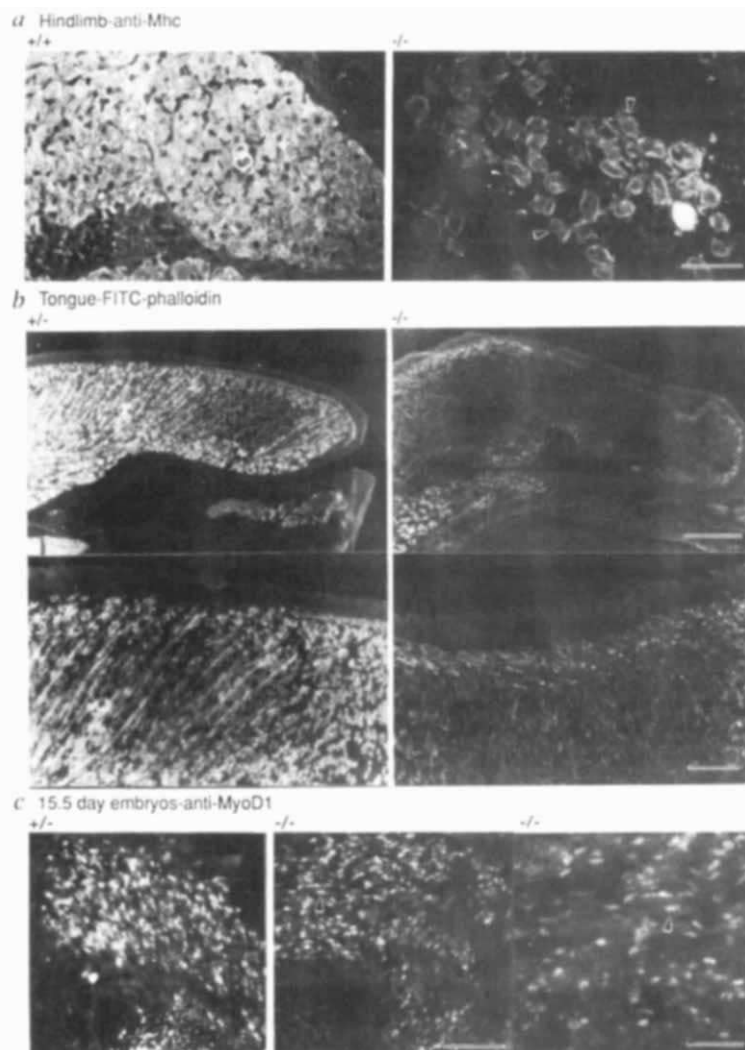
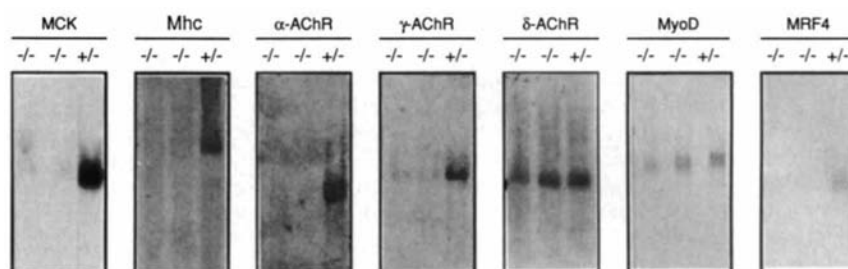


FIG. 5 Northern analysis of *myg^{ml}/myg^{ml}* mice using muscle-specific probes. Internal organs and heads of newborn mice were removed and total cellular RNA isolated from the carcasses²⁹ and hybridized with cDNAs for MCK³⁰, MHC³¹, AChR subunits α ³², γ ³² and δ ³⁴, MyoD³⁵ and MRF4 (ref. 36). Gel electrophoresis, hybridization and probe preparation have been described¹². Equal amounts of RNA were electrophoresed in each lane, as assessed by ethidium bromide staining of ribosomal RNA; quantification was achieved with a Molecular Dynamics Phosphorimager.



neonates, we conclude that normal levels of MyoD are not sufficient to overcome the severe muscle deficiency.

Discussion

Our results demonstrate that myogenin is required for skeletal muscle development *in vivo*. This function of myogenin is in sharp contrast to those of Myf5 and MyoD, neither of which is needed for formation of skeletal muscle in mice^{5,6}. Why myogenin and not the other myogenic bHLH factors? Myogenin transcripts appear at day 8.5 in developing somites, several hours after Myf5 transcripts⁷. Despite the fact that Myf5 transcripts are expressed before those of myogenin, Myf5 cannot compensate for the loss of myogenin activity. Myogenin must therefore play a unique role in skeletal muscle differentiation, presumably by acting specifically in one or more essential transcriptional events. This requirement for myogenin could reflect specificity in its temporal pattern of expression or in its intrinsic functions. In this regard, myogenin's non-conserved amino and carboxy termini distinguish it functionally from other myogenic factors²².

An intriguing feature of *myg^{ml}/myg^{ml}* animals is the presence of some, albeit less abundant, differentiated muscle fibres throughout the body. Staining of residual fibres by immunocytochemistry with anti-Mhc and with phalloidin (Fig. 4a, b), as well as ultrastructural observations (G. Decker and E.N.O., unpublished results), indicate that the muscle that forms in mutant pups has a normal sarcomeric organization. There are several potential explanations for the muscle observed in the *myg^{ml}/myg^{ml}* mice. First, a specific subset of embryonic myoblasts may not require myogenin for differentiation and may be capable of forming muscle even in the abnormal environment existing in the mutant mice. Myogenin may be required for differentiation of certain muscle fibre types, whereas other myogenic factors may be required for others. It is also possible that the appearance of muscle in the mutant mice may be a stochastic event, mediated by inherent fluctuations in the levels of other myogenic factors. If, for instance, Myf5 or MyoD levels exceed a threshold in a

random subset of muscle cells, they might substitute for myogenin.

The levels of muscle-specific mRNAs in the myogenin-mutant mice indicate that expression of different muscle-specific genes can be uncoupled. For example, transcript levels of MCK, Mhc and α - and γ -AChR subunits are dramatically reduced, suggesting either that they rely on myogenin for expression, or that the cells that express these transcripts are absent or reduced. Intriguingly, AChR δ -subunit expression seems to be unaffected by the absence of myogenin. Whether the δ -subunit gene is regulated by another member of the myogenic bHLH family, or whether its transcription is independent of these myogenic regulators remains to be determined.

Mice lacking myogenin have gross rib defects, but these abnormalities cannot manifest at the cellular level because bone and cartilage appeared histologically normal. In normal development, ossification occurs in the sternal cartilage except in the areas where ribs touch, forming ossified sternebra and intersternebra cartilage^{5,20,21}. In myogenin-mutant mice, either progression of the ribs to the sternal cartilage is delayed or ossification of the sternal cartilage is accelerated so that intersternebra cartilage becomes ossified. Because myogenin is expressed exclusively in skeletal muscle^{9,23}, the skeletal defects are likely to be secondary to the absence of skeletal muscle, resulting in a lack of tension on the developing skeleton.

Generation of *myg^{ml}/myg^{ml}* mice provides an important *in vivo* model for investigating the role of myogenic bHLH factors in muscle development. Here we have generated mice that are severely deficient in skeletal muscle as a result of the absence of a single regulatory gene. Despite the marked reduction in skeletal muscle, mutant mice survive fetal development and develop into neonates with identifiable morphology. We are at present attempting to identify where in the muscle differentiation pathway these mutant mice are blocked, and to characterize myoblasts lacking functional myogenin. These mice will also be useful for studying autoregulatory interactions among myogenic bHLH proteins during embryogenesis²⁴. □

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A type IIb model for supernova 1993J

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SPECTRAL analyses of supernovae have allowed them to be broadly divided into two classes, type I and type II. Supernova 1993J was identified¹⁻³ as a type II supernova following the detection of a weak hydrogen line in its early spectrum. But the optical light curve⁴ of SN1993J is atypical for this class of supernova, with the intensity rising to a second maximum after the initial outburst. The light curve around the second maximum more closely resembles that of the type Ib supernova 1983N (ref. 5), the progenitor of which may have been a helium star⁶. Here we show that the secondary brightening and subsequent decay of the light curve can be explained by the radioactive decay of ⁵⁶Co; combined with the early spectrum, this suggests that the progenitor may have been a red supergiant with an unusually thin hydrogen-rich envelope. If this model is correct, the spectrum will rapidly evolve from type II to type Ib, in which case SN1993J would be classified as a type IIb supernova^{7,8}. We suggest that the progenitor was in a binary system, and had lost most of its hydrogen envelope to the companion star before the explosion.

The light curve of SN1993J quickly reached the first maximum, declined in ~5 days, rose to the second maximum in ~10–15 days depending on the wavelength, and then declined again⁴. Such a light curve with two peaks is distinctly different from those of previously known type II supernovae which show either a long plateau (type II-P) or a relatively monotonic decline without a clear plateau (type II-L)⁹. The light curves of these type II supernovae have been well modelled with the explosion of massive red-supergiant stars (except for the blue supergiant progenitor of SN1987A). The explosion of such massive stars (≥ 8 solar masses ($M_{\odot}$)) is triggered by the gravitational collapse that forms a neutron star (or a black hole) and a shock wave. The shock wave propagates through the surface to heat up the extended hydrogen-rich envelope. Subsequent diffusive release of internal energy forms a light curve whose plateau duration depends on the mass and the initial radius of the progenitor's envelope^{10,11}. However, those ordinary massive red-supergiant models cannot explain the unusual light curve of SN1993J.

The important clue to the understanding of the nature of SN1993J has been provided by its bolometric light curve, which has been constructed⁴ using BVI photometry and bolometric corrections derived from models and SN1987A (the filled circles in Figs 1 and 2); the distance to SN1993J and the extinction are assumed to be 3.3 Mpc and $A_v = 0.4$ mag, respectively. Since the observed bolometric luminosity is the rate of total energy output integrated from the ultraviolet to the infrared bands, it can be compared with the theoretical bolometric luminosity to identify the energy source of the unusual light curve of SN1993J.

Because there is no resemblance between SN1993J and other type II supernovae, we extend the comparison to include type I supernovae. In Fig. 1 we plot the observed bolometric light curves of SN1993J (filled circles) and SN1983N (open circles) which is the best observed type Ib supernova⁵. It is striking that the light curve around the second maximum of SN1993J is in excellent agreement with that of SN1983N.

As the basic features of type Ib supernova light curves have been explained with the helium star models^{6,12-14}, we calculate the explosions of helium stars with masses of $M_a = 3.3$, 4 and

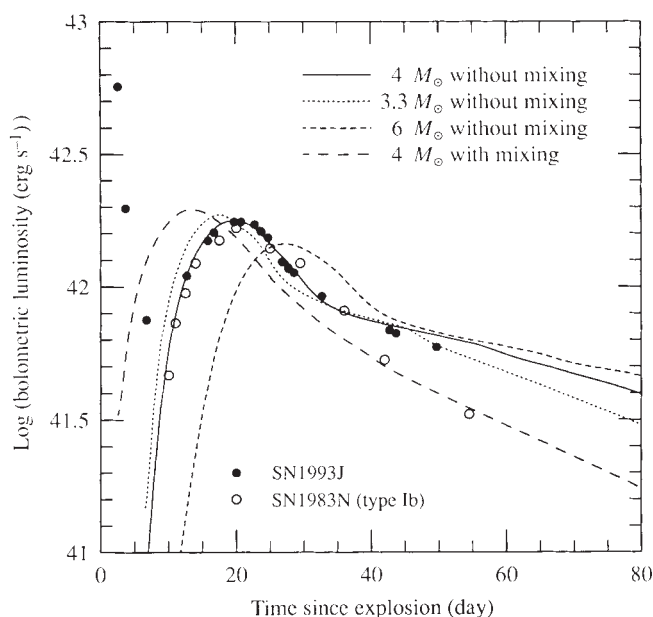


FIG. 1 The bolometric light curves of SN1993J (filled circles)⁴, the type IIb supernova 1983N (open circles)⁵, and the He star models with various masses and degrees of ⁵⁶Ni mixing.

$6 M_{\odot}$, which are presumed to form from the main-sequence stars with $M_{ms} = 13, 15$ and $20 M_{\odot}$, respectively¹⁵. These helium stars undergo gravitational collapse to form a neutron star and generate a shock wave as in type II supernovae. In our hydrodynamical calculations, parameters such as the neutron star mass and the initial energy given to the shock wave are chosen to produce the kinetic energy of explosion $E = 1 \times 10^{51}$ erg s^{-1} and $0.075 M_{\odot}$ of ⁵⁶Ni, which is synthesized in explosive nucleosynthesis behind the shock wave. Rayleigh–Taylor instabilities are found to take place during the shock propagation¹⁶, which induces some mixing of ⁵⁶Ni from the deepest layer to the helium envelope. In these models, the radioactive decays of ⁵⁶Ni $\rightarrow$ ⁵⁶Co $\rightarrow$ ⁵⁶Fe power the light curve of SN1993J around the second maximum. (Shock heating effect does not appear in the early light curve because of small initial radii.) If ⁵⁶Ni is well mixed through the surface, the second maximum is reached earlier because of earlier heating by the radioactive decays. Also, the light curve declines faster because of faster escape of γ -rays before thermalization occurs. Because the degree of ⁵⁶Ni mixing has not been accurately determined, we calculate two cases for each helium star⁶, one with nearly homogeneous mixing and one without mixing. In Fig. 1 are shown the theoretical curves for $M_a = 3.3$ – $6 M_{\odot}$, with and without mixing. It is seen that the light curves of the $3.3 M_{\odot}$ and $4 M_{\odot}$ models without mixing are in good agreement with SN1993J.

The resemblance between the observed and calculated light curves shown in Fig. 1 strongly indicate that the hydrogen-rich envelope of SN1993J contains a very small mass, so that it has little influence on the light curve shape powered by the radioactive decays in the core. If the hydrogen-rich envelope were as massive as $\sim 10 M_{\odot}$ (which is the case for a typical red-supergiant as described earlier), it would take ~ 100 days until the hydrogen-rich envelope becomes transparent. This is demonstrated by the dotted line in Fig. 2, for the model which has the $10.6 M_{\odot}$ envelope on top of the $4 M_{\odot}$ helium core.

To estimate how small the envelope mass should be, we construct an explosion model which has a helium core of $M_a = 4 M_{\odot}$ and a hydrogen-rich envelope of mass $M_{env} = 0.89 M_{\odot}$. (Its pre-

supernova radius and helium mass fraction are $R=300$ solar radii ($R_\odot$) and $Y=0.8$.) The hydrodynamics of explosion is simulated as in the helium star models and the model parameters are chosen to produce $E=1.2 \times 10^{51}$ erg s^{-1} and $0.075 M_\odot$ ^{56}Ni . Uniform mixing of ^{56}Ni in the helium core is assumed. The calculated bolometric light curve (the solid line in Fig. 2) shows two peaks as in SN1993J. The light curve around the first maximum is due to the shock heating of the envelope. Starting from the light curve minimum, the effect of heating by the ^{56}Co decay appears and produces the second peak. In this model, the hydrogen-rich envelope does not become transparent until the second peak, and the agreement with the SN1993J curve is not as successful as in the helium star models. This indicates that the envelope mass should be less than $\sim 0.9 M_\odot$. Further explorations of the correct model for SN1993J are underway for various combinations of the envelope models and core models¹⁷.

If the helium star models in Fig. 1 give the correct interpretation of the light curve around the second maximum, the mass of ^{56}Ni synthesized in SN1993J is $\sim 0.075 M_\odot$ with a factor of ~ 2 uncertainty (due to the estimates of the extinction and distance). Because of the thin hydrogen-rich envelope, SN1993J may be called a type IIb supernova⁷ as future spectral development may follow SN1987K, which changed from type II to type Ib (ref. 8). Depending on their abundances, line features of helium, oxygen, and calcium will appear due to radioactive excitation.

Possible progenitors that have a thin hydrogen-rich envelope include (1) an AGB star¹⁸, (2) a very massive star with the main-sequence mass $\geq 30 M_\odot$ (and $M_\alpha \geq 10 M_\odot$) that has lost most of the envelope in a red supergiant wind becoming almost a Wolf-Rayet star¹⁹, and (3) a binary star which has a $3\text{--}6 M_\odot$ helium core and lost most of its envelope to the companion star.

The AGB model would probably result in more like the monotonic decline type supernova (type II-L) because of the large initial radius as well as very small total ejecta mass^{11,20}, although a helium rich envelope model may not be precluded¹⁸. To distinguish the other two models, the mass of the helium core can be constrained from the date of the second maximum, which is ~ 20 d. For more massive helium stars, heat generated by the radioactive decay takes longer to diffuse out by means of electron scattering. Accordingly the helium star models with masses $\geq 6 M_\odot$ reach the light curve maximum after day 25. Even with the large scale mixing of ^{56}Ni , the $6 M_\odot$ model shows a maximum at day 20 (ref. 6). Therefore, the helium core mass would be in the range of $3\text{--}6 M_\odot$, which favours the binary scheme. Further information on the decline rate of the light curve would provide more accurate determination of the helium core mass and the degree of the ^{56}Ni mixing as seen in Fig. 1. For longer terms the decline of the bolometric light curve will be slowed down by two effects. First, the time scale of energy emission due to recombination of ions will become longer than that of energy input from the radioactive decay (which mostly goes into ionization²¹.) Second, possible pulsar activity may heat up the ejecta.

These arguments suggest that the progenitor of SN1993J was in a binary system and lost most of its envelope to the companion star. The progenitor may have become detached from the Roche lobe as a result of the reversal of the mass ratio. In the binary scheme, the asymmetry of SN1993J inferred from the polarization²² and line features²³ may be naturally explained by the binary interaction.

The hydrogen-poor model for SN1993J would lead to several interesting predictions depending on the chemical composition in the core. (1) At a distance of 3.3 Mpc, the Compton Gamma Ray Observatory (CGRO) could detect the line γ -rays from supernovae of type Ia, Ib and Ic, but not from ordinary type II-P (refs 24–26). If SN1993J has a small enough hydrogen-rich envelope, produced more than $\sim 0.15 M_\odot$ ^{56}Ni , and underwent mixing, the observations of line γ -rays from SN1993J with CGRO could be just possible around day 100. (2) SN1993J could form a helium-rich supernova remnant, which might look

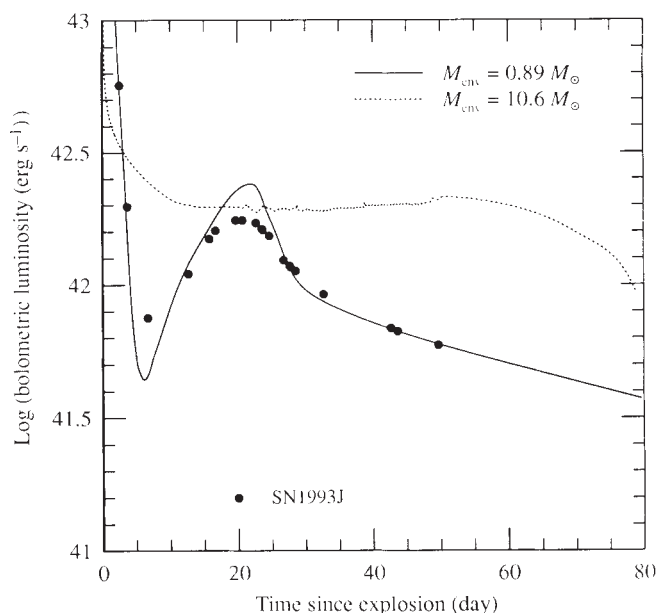


FIG. 2 The calculated bolometric light curves for two models, with envelopes of different masses M_{env} on top of the same $4 M_\odot$ helium core. The solid and the dotted lines show the cases with $M_{\text{env}}=0.89$ and $10.6 M_\odot$, respectively. The bolometric light curve of SN1993J is shown by the filled circles⁴.

like the helium-rich Crab Nebula²⁷ if most of heavy elements form dust grains in the remnant. SN1054 was visible during the day for about a month²⁸, which could happen with SN1993J if its light curve continues to decline.

In summary, we suggest that SN1993J is a supernova that occurred in a binary system and may be classified as a type IIb supernova.

Note added in proof: After this paper had been submitted Filippenko and Matheson²⁹ reported the emergence of prominent helium line features in the spectra of SN1993J, supporting our prediction for the spectral development from type II to type Ib. □

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The progenitor of supernova 1993J: a stripped supergiant in a binary system?

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SUPERNOVA 1993J in the spiral galaxy M81 is the brightest supernova since SN1987A and, like the latter, appears to be another 'peculiar' type II supernova. The available photometry^{1,2} of the supernova region before the explosion requires the presence of at least two supergiants (one of early spectral type and the other of late type), but the actual progenitor has yet to be identified. Here we show that the explosion of a late-type supergiant can explain the initial sharp peak in the supernova light curve, provided that the star had lost almost all of its hydrogen-rich envelope before the explosion. In our model, the secondary brightening of the supernova, ~10 days later, is then a consequence of the radioactive decay of ⁵⁶Ni (and subsequently ⁵⁶Co) produced in the explosion. The progenitor could have lost its hydrogen-rich envelope either in a strong stellar wind or, as seems more likely, through mass transfer to a companion star. In the latter case, the companion should reappear after the supernova photosphere has receded, the system having become a binary composed of a neutron star with a massive stellar companion.

The early light curve of SN1993J is characterized by a very sharp initial peak (lasting for less than ten days) followed by a less rapid secondary brightening, which was qualitatively similar to the secondary brightening observed in SN1987A. The unusual initial peak and the absence of an extended plateau phase relegate SN1993J to the category of peculiar supernovae. Humphreys *et al.*¹ have identified a candidate progenitor consistent with the position of the supernova. Combining their ultraviolet-blue-visible-red (UBVR) photometry with the infrared (I) magnitude obtained by Blakeslee and Tonry², they concluded (personal communication) that the colours of the apparent progenitor require the presence of at least two bright stars. The results of these fits are sensitive to the assumed visual extinction, A_V , and also to the assumed reddening law. Nevertheless, the presence of two supergiants of comparable brightness is required for all values of A_V for which good fits can be found ($A_V \lesssim 2$). One star is an early-type supergiant (most likely a late-B to early-A supergiant), the other a late-type supergiant (most likely a G to early-K supergiant). The bolometric magnitudes (M) of both stars are in the range of -6 to -8 , with best-fit values of -7 to -7.5 (for an assumed distance modulus, $(m-M)_0$, of 27.6). These best-fit values imply main-sequence masses of ~ 15 solar masses ($M_\odot$) but the masses could have been as low as $8 M_\odot$

and as large as $20 M_\odot$ (larger masses would require very large bolometric corrections and/or a very large value for $A_V + (m-M)_0$).

The image of the candidate progenitor appears extended on some plates². This suggests that, at the distance of M81, the two stars are separated by at least a few light years and that it is unlikely that the two stars are gravitationally bound to each other (that is, form a binary system). However, this does not rule out that the progenitor was in a close binary, because the presence of one or more additional stars is consistent with the photometric fits. In fact, this is *a priori* quite probable, because the majority of massive stars are believed to be members of binary systems (see for example, refs 3, 4). The likely presence of two supergiants in the field of SN1993J raises the question of which of the two stars was the actual progenitor. An early-type supergiant is an unlikely candidate for a (bright) type II supernova^{5,6} (although SN1987A violated this theoretical expectation), whereas an ordinary red supergiant probably produces a more normal type II supernova^{5,6}. Furthermore, the spectral type of the late (G to early K) component is significantly earlier than the typical spectral type of a massive red supergiant (early to mid M; ref. 7). As we shall show below, however, a red supergiant progenitor can account for the early light curve, provided that it had lost almost all of its hydrogen-rich envelope before the supernova event (that is, provided it was a 'stripped' supergiant).

We previously carried out a detailed study of supernovae with stripped red-supergiant progenitors (which we refer to as type II (stripped) supernovae; see refs 8, 9 and J.J.L.H. *et al.* manuscript submitted). We found that supernovae of this type differ from ordinary type II-P supernovae (which have red-supergiant progenitors with large hydrogen-rich envelopes) in two important respects. (1) The plateau phase of the light curve, which corresponds to the phase where the hydrogen-recombination front

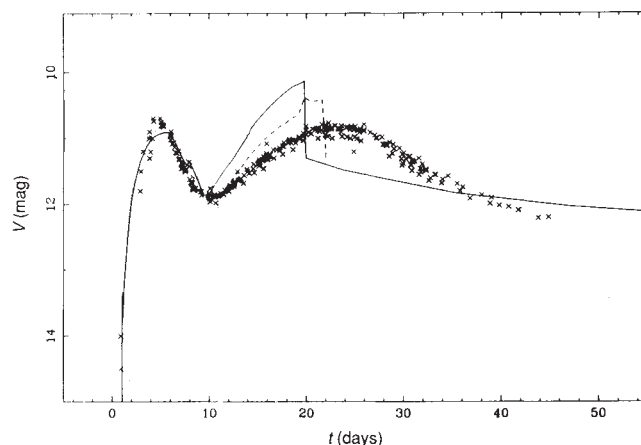


FIG. 1 Comparison of theoretical visual light curves with observations of SN1993J (apparent visual magnitude (V) plotted against time (t) in days since the explosion; 1993 March 26.0 UT corresponds to $t=0$). The crosses represent observed V magnitudes, as compiled by T. Kato (personal communication). The theoretical light curves assume a distance modulus to M81 of $(m-M)_0 = 27.6$ and a visual extinction $A_V = 0.8$ mag. The bolometric corrections were calculated by assuming a hydrostatic stellar atmosphere and by adopting a constant effective temperature of 5,000 K after the secondary peak. The progenitor in all three cases, is a red supergiant (of spectral type K) which had an initial main-sequence mass of $15 M_\odot$, but had lost all but $0.2 M_\odot$ of its hydrogen-rich envelope. The solid curve shows the visual light curve for a model with an explosion energy $E = 9 \times 10^{50}$ erg plus energy from the radioactive decay of $0.15 M_\odot$ of ^{56}Ni (produced in the explosion) and its decay product, ^{56}Co . The dotted curve represents a model with $E = 10^{51}$ erg and no nickel. The third model (shown as a dashed curve) is similar to the first model, except that the opacity law has been modified to smooth the secondary rise of the light curve (using the formalism in ref. 16).

recedes through the ejecta, becomes shorter as the residual envelope mass of the progenitor star decreases. When the envelope mass is less than $\sim 0.5 M_{\odot}$, there is no distinct plateau phase, and the early light curve is characterized by a single, well-defined peak. (2) If radioactive nickel is produced during the supernova (this depends on the original mass of the progenitor and the details of the explosion mechanism), the energy released from the decay of nickel (and subsequently cobalt) produces a short, but visible, secondary maximum. The observed light curve of SN1993J seems to show both of these predicted features. It is also worth noting that the spectral type of a stripped supergiant will be significantly earlier than the spectral type of a red supergiant with a large hydrogen-rich envelope (spectral type K rather than M), which is in excellent agreement with the photometric constraints discussed above.

We therefore performed a series of hydrodynamical calculations to investigate the possible range of parameters for a stripped-supergiant progenitor of SN1993J. (Technical details of our calculations can be found in the references listed above.) Our main result is that the observed early visual light curve of SN1993J can be fitted very well with the explosion of a progenitor star with a main-sequence mass of $15 M_{\odot}$, which suffered severe mass loss and had a residual hydrogen envelope mass of $\sim 0.2 M_{\odot}$ and an explosion energy $E \approx 9 \times 10^{50}$ erg. Furthermore, if it found that the light curve of SN1993J after ~ 40 days is following the exponential decay of cobalt, then both the secondary peak of the light curve and the exponential decay can be understood as the result of $\sim 0.1 M_{\odot}$ of nickel that was produced in the supernova and confined to the inner regions of the ejecta (the exact amount of nickel depends sensitively on the assumed reddening and the distance to M81).

In Fig. 1 we display the observed visual light curve of SN1993J and compare it with a few theoretical light curves we have calculated. The first peak in the light curve within ~ 10 days after the explosion is mostly produced by the initial expansion of the supernova ejecta (that is, it took only ~ 10 days for all of the hydrogen to recombine). If no nickel had been produced in the supernova, the light curve would have dropped rapidly and faded away after the initial peak (the dotted curve in Fig. 1). With the additional energy from the radioactive decay of $0.15 M_{\odot}$ of nickel, however, the light curve (the solid curve in Fig. 1) then rises to a secondary maximum (in 25 days) and drops to follow the exponential decay curve of cobalt. (The cause of the secondary peak is very similar to that inferred for the secondary peak in SN1987A.) It is apparent that our calculated light curve rises more sharply to the secondary maximum and fades more rapidly thereafter than is observed. This is similar to the situation in SN1987A and is caused by several effects we did not consider, in particular the effects of Doppler broadening on the opacities and the effects of mixing of hydrogen and nickel through the ejecta during the explosion. (A detailed discussion of these issues and a more detailed comparison of our models with the observations will be published elsewhere; J.J.L.H. *et al.*, manuscript in preparation.)

Our explosion model for SN1993J makes two firm predictions for the immediate further evolution of the supernova. (1) Because of the low mass of the hydrogen-rich ejecta, the late supernova spectrum should resemble that of a type Ib/Ic supernova (that is, the supernova should transform itself from a type II to a type Ib/Ic supernova similar to SN1987K; ref. 10). At the time of submission of this letter, this prediction may already have come true¹¹. (2) There should, in the near future, be a resurgence of the X-ray flux from SN1993J, because the γ -rays produced by the decay of cobalt should soon be able to escape as X-rays (the degradation of their energies is due to multiple Compton scatterings as the photons diffuse through the ejecta). Such X-rays have been observed in SN1987A, but, at the distance of M81, this resurgence may not be detectable.

There are two principal evolutionary schemes that can explain the existence of a progenitor with a small hydrogen-rich envelope.

The first is one in which the progenitor lost most of its hydrogen-rich envelope in a strong stellar wind. Theoretical models of stellar winds in post-main-sequence stars¹² indicate that an initial main-sequence mass $\geq 25 M_{\odot}$ would then be required. Such masses are higher than the probable mass implied by the photometric fits (although one cannot exclude such masses with a high degree of confidence). Moreover, this single-star scenario would require that the initial mass of the progenitor fell within a very narrow range, because otherwise the progenitor would have been either a normal red supergiant or a Wolf-Rayet star.

A more probable scheme is one in which the progenitor was a member of a close binary system and underwent stable case C mass transfer^{13–15}. In this case, the progenitor (the primary of the binary) fills its Roche lobe while on the asymptotic giant branch (that is as a red supergiant after helium core-burning) and loses most of its envelope in a dynamically stable mass-transfer phase (see ref. 15 for details). The companion star will have accreted a significant fraction of the transferred mass and thereby become substantially more luminous (in fact, it may have become more luminous than the primary). When the mass in the hydrogen-rich envelope has decreased below a certain critical value ($\sim 0.3 M_{\odot}$ for a progenitor mass of $\sim 15 M_{\odot}$), the envelope of the progenitor will shrink significantly, so that the star will no longer fill its Roche lobe and mass transfer will cease. The primary will initially still have the appearance of an ordinary red supergiant but will have only a residual hydrogen-rich envelope with a mass of at most a few tenths of a solar mass. Subsequently, the residual envelope will be slowly eroded by a stellar wind; however, even for a mass-loss rate $\dot{M} \approx 10^{-5} M_{\odot} \text{ yr}^{-1}$, it will take several times 10^4 yr before the envelope has been completely lost. Because this timescale is of the same order as the remaining evolutionary timescale after the case C mass-transfer phase, a remnant hydrogen-rich envelope may still be left at the time of the supernova explosion. Thus, this scenario would avoid the fine-tuning required in a single-star scenario for a stripped-supergiant progenitor; it only requires case C mass transfer, which occurs for a fairly large range of orbital periods (between ~ 1 and ~ 30 yr for typical binary parameters).

The binary scheme described above makes a number of testable predictions. The companion of the original binary system should initially disappear from view below the photosphere of the supernova, but it should ultimately reappear after the photosphere has receded sufficiently. We emphasize that, because of the mass transfer, the companion may have been more luminous than the progenitor. In this case, neither the early-type supergiant nor the late-type supergiant would have been the progenitor and both stars should still be present after the explosion. Even before the optical reappearance of the companion, it may make its presence known as an additional energy source in the light curve (provided that the supernova did not produce a luminous Crab-like pulsar). Also, the binary is likely to remain bound after the supernova explosion, because much less than half the total mass of the system should have been ejected in the explosion. Hence, the system should now have become a binary composed of a neutron star (or black hole) in a significantly eccentric orbit (with an eccentricity $e \approx 0.1$ – 0.5) with a massive stellar companion. The most straightforward confirmation of the proposed scheme would be the detection of the collapsed remnant as a pulsar displaying orbital Doppler shifts in its pulse period.

Finally, we note that our binary scheme suggests a possible evolutionary link between this supernova and SN1987A. In this scheme, the companion accretes several solar masses from the primary. If this accretion takes place after the main-sequence phase of the companion (as would be the case if the companion is a bright supergiant), the future evolution of the companion will be drastically altered. The companion is then likely to explode as a blue supergiant (in 10^5 to 10^6 yr from now) and this second supernova will resemble SN1987A (ref. 15).

Note added in proof: After this work had been completed, we became aware that two other groups, K. Nomoto *et al.*¹⁷ and S. E. Woosley *et al.* (preprint) independently arrived at a model for SN1993J which, in many respects, is similar to the one presented here. □

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Evidence for surface heterogeneity on Titan

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UNLIKE all other planetary satellites, Saturn's moon Titan has a massive atmosphere^{1–8}. At visible wavelengths, a thick stratospheric haze hides the surface from view. The emission from Titan in the infrared is largely from methane, nitrogen and hydrogen also in the stratosphere¹. In the near-infrared, however, the extinction from haze decreases, and narrow windows exist in which the atmosphere absorbs only weakly^{9–14}, and through which we might therefore catch a glimpse of the surface. Within two of these windows, Lemmon *et al.*^{15,16} recently observed a difference in Titan's albedo when the satellite was at eastern and western elongation with respect to Saturn. Although these observations could be taken to imply that Titan's surface is heterogeneous (and therefore is not covered by a global methane–ethane ocean as predicted previously⁷), they could also be explained by transient clouds. Here I present observations from two more rotational periods which

record the same albedo difference, indicating that the heterogeneity is most unlikely to be associated with transient features and must be intrinsic to the surface. These results also imply that Titan is locked in a synchronous orbit about Saturn.

Ultraviolet, visible, infrared and radar observations of Titan have revealed an atmosphere consisting of nitrogen (1.44 bar), methane (~0.05 bar), hydrogen (~0.006 bar), trace amounts of many organic compounds, and a ubiquitous haze^{1–6}. In the near-infrared, at several spectral regions, Titan's atmosphere appears optically thin^{10–14,17,18}. Evidence for seeing Titan's surface at these windows would be provided if Titan displayed a variable albedo that correlated with its rotation period. Titan's rotation period is unknown; however, it is probably synchronized with Titan's orbital period, because this configuration (having the lowest tidal energy) is observed for most satellites¹⁹. In this case, opposite hemispheres face the Earth when Titan is at opposite elongations in its orbit about Saturn.

Using NASA's Infrared Telescope Facility on Mauna Kea, equipped with the Cooled Grating Array Spectrometer²⁰, I observed Titan at opposite elongations in 1989 and 1990 (Table 1). I found that Titan's albedo at its eastern elongation was $11 \pm 10\%$, $20 \pm 10\%$, $13 \pm 7\%$, and $8 \pm 6\%$ greater than that at its western elongation at 4,900, 6,300, 7,800 and 9,300 cm^{-1} , respectively (Figs 1, 2). At 4,900 and 9,300 cm^{-1} , these differ-

TABLE 1 Observations and albedo ratios

Observations*† (this work)					
Wavenumber (cm ⁻¹)	Date (UT)	Nearest elongation	Time from elongation	Air mass‡	
4,000–5,100	1990 July 25 09 h	Eastern	+51 h	1.33–1.40	
4,000–5,100	1990 July 15 10 h	Western	–2 h	1.37–1.47	
5,600–6,800	1989 July 5 10 h	Eastern	+9 h	1.55–1.65	
5,600–6,800	1989 July 13 10 h	Western	+5 h	1.32–1.35	
7,500–9,400	1989 July 5 09 h	Eastern	+8 h	1.40–1.44	
7,500–9,400	1989 July 13 09 h	Western	+4 h	1.38–1.41	
Ratio of Titan's leading to trailing hemisphere albedos					
Wavenumber (cm ⁻¹)	Observations§ (this work)		Lemmon <i>et al.</i>		Ratio (haze removed)
	Ratio	Date	Ratio	Date	τ_{haze}
4,900	1.12 ± 0.04	1990	—	—	0.14–0.17
6,300	1.20 ± 0.10	1989	—	—	0.18–0.20
7,800	1.13 ± 0.07	1989	1.22 ± 0.03	1992	0.23–0.50
9,300	1.09 ± 0.03	1989	1.14 ± 0.03	1992	0.23–0.50

* Resolving power: 138–120, 200–171 and 140–120 at 4,000–5,100, 5,600–6,800 and 7,500–9,400 cm^{-1} , respectively.

† Calibration stars: SA0162229 and SA0188337 in 1989 and 1990, respectively.

‡ The air mass, $1/\cos \theta$, where θ is the zenith angle, is a measure of the relative pathlength viewed through the Earth's atmosphere during the observations.

§ At 4,900 and 9,300 cm^{-1} , spectrally degraded observations were used to compute the ratio. Detailed dates in upper part of Table.

|| Detailed dates given in text.

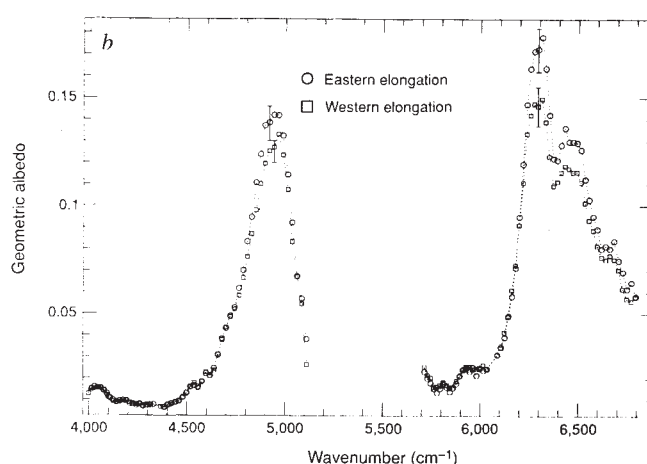
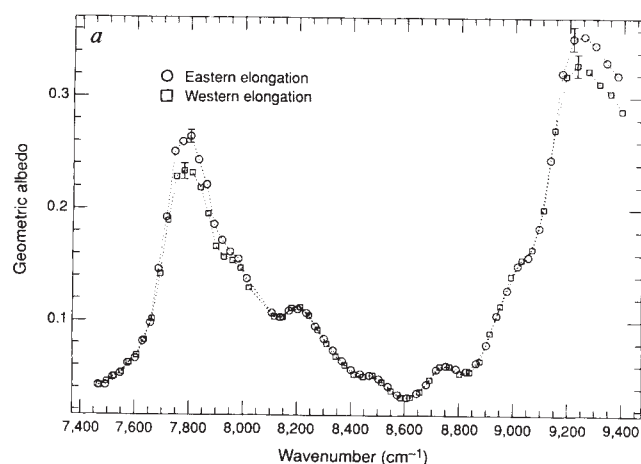


FIG. 1 Geometric albedo of Titan taken at opposite elongations in its orbit with respect to Saturn. *a*, Higher wavenumber; *b*, lower wavenumber. Titan's albedo is low where CH_4 absorption is strong (and attenuates incident solar radiation) and high where CH_4 absorption is weak enough that solar radiation penetrates below the tropopause (30 km)¹². The signal-to-noise ratios are 33 (*a*) and 17 (*b*). $\pm 1\sigma$ error bars resulting from instrumental noise are smaller than the spectral points at low albedos. Representative error bars are shown for the high-albedo spectral points. Titan's flux was calculated from averages of several observations, divided by a calibration star (Table 1), and multiplied by the black-body spectrum of the appropriate temperature. This flux was then divided by a single solar spectrum²⁹ convolved to the same spectral resolution to obtain Titan's albedo. The spectral regions of interest occur where the sun's radiation is not expected to vary. If

the seeing is bad for small periods within an observation, Titan's image can disappear from view, and only a fraction of Titan's flux is integrated during the observation. I therefore normalized Titan's flux by a factor that takes into account the problems with seeing and tracking. This factor was derived by matching Titan's spectra at the wavenumbers where Titan's albedo is not expected to change, at 4,000–4,500 cm^{-1} , 5,600–6,200 cm^{-1} and 8,200–8,800 cm^{-1} . At these wavenumbers, Titan's albedo is a manifestation of the CH_4 abundance and haze distribution between 40 and 130 km altitude¹², which depend primarily on the temperature at the cold trap and the solar ultraviolet flux incident on Titan's atmosphere. The hemispherical averages of these properties are not expected to change in the course of seven days between observations.

ences are only marginally significant. To improve the signal-to-noise ratio, the spectral resolution was degraded by a factor of four, resulting in albedo differences of $12 \pm 4\%$ and $9 \pm 3\%$, respectively. These observations display the same albedo heterogeneity recently reported^{15,16}; Titan's albedo at 9,300 and 7,800 cm^{-1} , on 13 September 1992 (eastern elongation), was larger by $14 \pm 3\%$ and $22 \pm 3\%$ than that observed on 19 July 1992 (western elongation)^{15,16}. In all cases, the variations in Titan's albedo occur precisely at the spectral regions where methane absorption is weakest. In these atmospheric windows, Titan's albedo is a manifestation of its surface albedo and/or CH_4 clouds, if such clouds exist below the tropopause^{5,10,12,13}.

On a planetary body with a surface (unlike Jupiter), clouds are expected to be transient features, unless they are associated with topography. The reproducible variation in Titan's albedo, which is correlated with orbital position, indicates therefore a non-uniform surface brightness or the presence of a stationary cloud pattern associated with topography. In either case, the reproducibility of the observations suggests that Titan has a heterogeneous surface and synchronous rotation within 2.7 h.

The spectral qualities of the materials that cause the variation in Titan's albedo can be derived by dividing the spectrum of Titan's leading hemisphere by that of the trailing hemisphere (Fig. 3), observed at eastern and western elongations respectively. This ratio depends weakly on Titan's haze and strongly on possible clouds and the surface albedo. Haze does not add fine features to Titan's spectra and instead affects the overall continuum. Near-infrared and visible observations^{10,12,18} indicate that Titan's haze has near-infrared optical depths (τ_{haze}) in the range shown in Table 1. To study the spectral qualities of the clouds and surface below Titan's haze, the effects of haze were removed from the spectrum using a two-layer model of Titan's atmosphere. The top layer corresponds to Titan's atmosphere above 30 km altitude; this layer models Titan's haze following the particle sizes and distributions derived from ref. 18. The bottom layer is the unknown; it corresponds to the atmosphere below 30 km and is a manifestation primarily of possible clouds and Titan's surface. I have modelled each of Titan's spec-

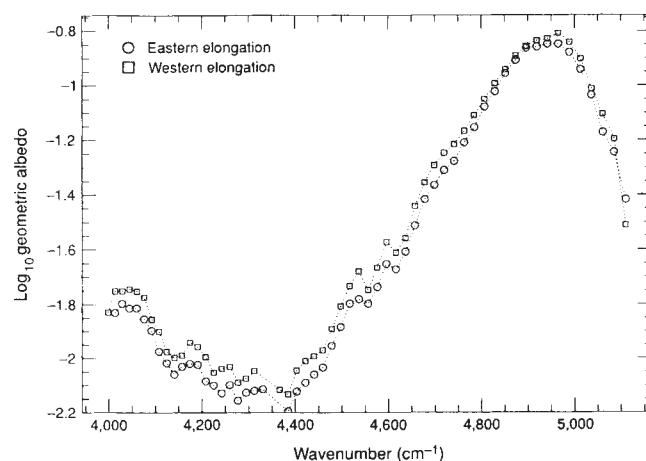


FIG. 2 Log plot of Titan's geometric albedo, taken over a restricted wavenumber range and normalized to match the highest albedo points, as opposed to the low albedo points used in Fig. 1. The data at eastern and western elongations are shown to disagree by the same percentage at 4,360 and 4,600 cm^{-1} , wavenumbers that sample different altitude regions in Titan's atmosphere (110 and 60 km, respectively¹²). The possibility that a simultaneous change in Titan's atmosphere occurs at these two different altitudes is improbable, lending further support for the normalization procedure used in Fig. 1.

tra (Figs 1 and 2) at 4,900, 6,300, 7,800 and 9,300 cm^{-1} and derived the albedo for the unknown bottom layer. I then divided the leading by trailing hemisphere albedos of these bottom layers. This way I was able to extract the effects of Titan's haze and compute the ratio of Titan's leading to trailing hemisphere albedos resulting from the clouds and surface below the haze (presented in Table 1).

The fact that there is a wavelength dependence in these haze-corrected ratios suggests that we are seeing the effects of an

absorbing gas, clouds or the satellite's surface. The optical constants of CH_4 and C_2H_6 ice^{21,22} indicate that CH_4 and C_2H_6 clouds would be highly reflective and grey at 4,900, 6,300, 7,800 and 9,300 cm^{-1} . Methane gas is the only absorbing material in the atmosphere that might account for the wavelength dependence of the haze-corrected ratio. Available laboratory spectra of methane, however, do not indicate this wavelength dependence. Instead, laboratory data indicate that if solar radiation penetrated an extra several kilometres into Titan's atmosphere on one hemisphere, we might observe at most a slight, wavelength independent difference in Titan's albedo²³. Another source of absorption is necessary. The only other available absorber is Titan's surface.

The haze-corrected ratio of Titan's leading to trailing hemisphere albedos is consistent with the ratio of reflectivities of a variety of possible surface materials. For example, it agrees with a Titanian leading hemisphere similar to that of Iapetus²⁴, composed of H_2O ice mixed with impurities that absorb strongly at larger wavenumbers. Another explanation however is a surface that has strong CO_2 absorption on the leading hemisphere. Given the uncertainties and range of possible surface materials, the broad structure of the ratio is not diagnostic of the surface composition.

The fine structure of Titan's spectra within the spectral windows may allow a better estimate to be made of the composition of Titan's surface. The largest region available inside a window lies between 6,200 and 6,700 cm^{-1} (Fig. 3). At 6,300–6,600 cm^{-1} , a slope exists that may define the wing of a spectral feature at 6,570–6,700 cm^{-1} . This possibility must be verified with additional observations having better signal-to-noise ratios. Such a feature corresponds closely to the H_2O ice absorption²⁴ seen in the ratio of spectra of Europa's leading to trailing hemispheres. The continuum of Titan's near-infrared spectrum appears consistent with a surface containing water ice and dark impurities¹². In addition, water ice is observed on the surfaces of most satellites in the outer solar system²⁴. Titan's surface, however, lies below a rain of organic material and is expected to be covered with sediments. Among the many unknown aspects of Titan's lower atmosphere is its weather; a C_2H_6 – CH_4 rain could, for example, expose the ice and rock 'bedrock' by washing solid sediments off highland slopes.

Liquid CH_4 and C_2H_6 are thought to be major components of Titan's heterogeneous surface^{7,8}. This follows from considerations of the fate of CH_4 in Titan's atmosphere; stratospheric CH_4 is rapidly destroyed by solar ultraviolet photolysis⁸, and thus requires a recent source. CH_4 – C_2H_6 oceans⁷, as well as volcanic outgassing of CH_4 , and aquifers²⁵, have been proposed as sources for atmospheric CH_4 . Moreover, oceans are also expected because C_2H_6 , the main by-product of CH_4 photolysis⁷, condenses out of Titan's atmosphere and forms a liquid at Titan's surface temperature (95 K). Assuming the present ethane production rate, enough has been produced in the past 10^9 years to yield more than 200 m of liquid ethane on the surface²⁶. If Titan's topographic relief is similar to that of the icy Galilean satellites, that is several hundreds of metres, liquid could cover a considerable fraction of the surface. Titan's radar reflectivity however indicates that if Titan was largely covered by oceans, they must be less than 200 m deep²⁷. Additional information comes from considerations of tidal dissipation of Titan's orbit about Saturn. Sagan and Dermott²⁸ have concluded that the large eccentricity of Titan's orbit indicates that if an ocean existed, it must be global and greater than 400 m deep.

We are left with a problem in reconciling the inferred production of liquid ethane, radar and near-infrared observations, and Titan's eccentric orbit. Both radar and near infrared observations are consistent with a partly dry surface with deep CH_4 – C_2H_6 oceans or a mostly dry surface where methane is supplied by volcanic degassing of the interior or from hydrocarbon 'aquifers'. The first suggestion is not consistent with Titan's large eccentricity. The second does not explain what happens to the

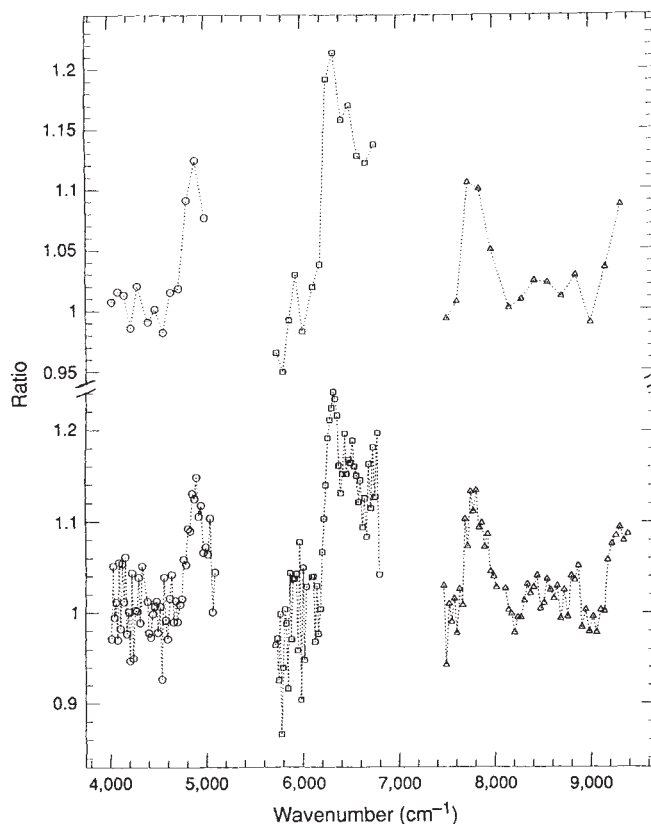


FIG. 3 Titan's leading hemisphere infrared spectrum (observed at eastern elongation) divided by Titan's trailing hemisphere infrared spectrum (observed at western elongation) at full spectral resolution (bottom curve) and at a spectral resolution degraded by a factor of four (top curve). The solid line corresponds to the ratio of Europa's leading to trailing side spectra (Clark *et al.*²⁴), scaled to 0.99 at 6,557 cm^{-1} .

large mass of precipitated liquid C_2H_6 . The nature of Titan's surface remains unresolved. However the observations presented here, combined with those of Lemmon *et al.*^{15,16}, strongly suggest that: (1) future ground-based observations will allow for investigations of Titan's surface and rotation period; (2) the Imaging Science Subsystem (ISS) on board the Cassini mission will be capable of mapping Titan's surface at 9,300 cm^{-1} ; and (3) the Visible-Infrared Mapping Spectrometer (VIMS) on board Cassini will be able to map Titan's surface at 4,900, 6,300, 7,800 and 9,300 cm^{-1} with a spatial resolution of up to 1 km. □

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Radial deformation of carbon nanotubes by van der Waals forces

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THE discovery of carbon nanotubes^{1,2} has stimulated many theoretical studies of their physical properties^{3–12}, and their bulk synthesis¹³ should soon make possible experimental measurements of these properties¹⁴. All studies so far have assumed that the nanotubes have perfect cylindrical symmetry. Here we show that van der Waals forces between adjacent nanotubes can deform them substantially, destroying this cylindrical symmetry. We present transmission electron microscopy images of two adjacent aligned tubes, about 100 Å in diameter, which show flattening of the tubes along the contact region. Calculations on two-layer nested nanotubes indicate that these deformations can be explained on the basis of van der Waals interactions. We predict that these effects should be observable at least for tubes as small as 20 Å in diameter, and suggest that they may have a significant influence on the tubes' physical properties.

The importance of the van der Waals interaction and its role in the energetics and elastic deformation of 'molecular' systems have been extensively discussed¹⁵. For example, the Lennard-Jones (L-J) 6–12 potential with appropriate parameters has been used to describe the van der Waals interaction between C atoms in fullerene solids such as C₆₀(s) and C₇₀(s), between C₆₀ and graphite, and between planes of graphite. Empirical approaches that consist of summing over all unique C atom pairs have been essentially successful in their calculation of (low-pressure) properties of f.c.c. C₆₀(s), such as the lattice energy, zero-pressure bulk modulus, intermolecular separation, and bulk elastic properties^{16–20}; the same can be said for calculation of the (low-pressure) cohesive energy, interplanar separation, zero-pressure bulk modulus, and elastic constants of graphite²¹. Using a similar approach, the binding energy between an isolated C₆₀ molecule and a graphite plane has also been calculated and is surprisingly large, about 1 eV²². In these cases, the molecules or planes could be treated as rigid, and good agreement with experiment is obtained for a variety of properties even with this approximation. There are many other systems, however, in which large deformations in shape will occur, such as a liquid drop or a spherical vesicle, which each distort on a flat surface to become truncated spheres, or two adhering spherical

vesicles¹⁵. Such 'molecular' systems are substantially larger, and more flexible, than C₆₀.

We performed transmission electron microscopy (TEM) of carbon growths that contain nanotubes, produced as described in ref. 13. In the TEM images we discovered two (nested) nanotubes that are almost perfectly aligned parallel to each other. These are shown in Fig. 1. One clearly sees sets of 10, 22 and 12 fringes. Thus the image represents two adjacent tubes, one of 10 nested layers and one of 12. The inner set of 22 fringes is strikingly more intense than the two sets of outer fringes. The diffraction conditions were such that small differences in interlayer (*d*) spacings, discussed further below, do not explain why the inner fringes are so much darker than the outer fringes. We can be sure Fig. 1 represents two adjacent tubes because the sum of outer fringes is equal to the number of inner fringes.

The two adjacent tubes A and B extend well beyond the section indicated in Fig. 1. There is a juncture where a 'loop' internal to tube A is apparent, having three layers (such internal loops appear in many nanotubes, and have been mentioned in the literature: see for example refs 1 and 2). At this juncture and beyond, the number of inner fringes increases to 25, and the number of outer fringes for the A tube increases to 13. Thus, the sum of outer fringes (13 + 12) is again equal to the sum of inner fringes, which confirms that we were viewing two adjacent nanotubes.

We propose that these large fringe intensity differences arise from a flattening of the nanotubes where they are pressed together by the van der Waals interaction. Further evidence of this is the compression of the interlayer spacings in the interface region. We have measured these directly from microphotometer scans of the high-resolution TEM negative. A typical scan perpendicular to the long axis of each tube is shown in Fig. 2. The intensity differences evident to the eye in Fig. 1 are quantitatively

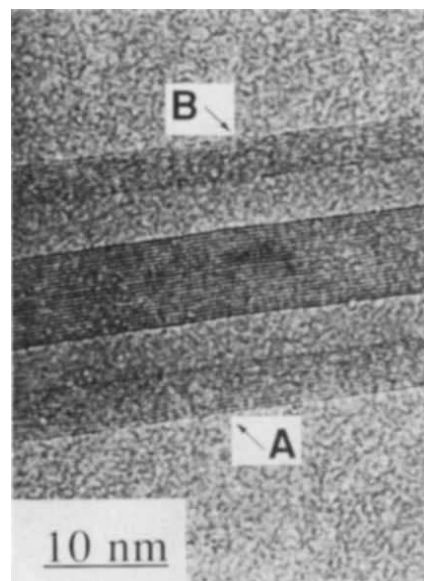


FIG. 1 High-resolution TEM micrograph of two (nested) adjacent nanotubes obtained at Scherzer defocus using a JEOL 2000FX TEM operated at 200 kV. Nanotube A contains 10 layers, and B contains 12 layers. The sum of the outer fringes is 22, which is equal to the sum of the inner fringes. Interlayer spacings are determined by calibrated microphotometry of the TEM negative, as described in detail in Fig. 2 legend and in the text. The outer diameter of A is 105 Å and the average interlayer spacing is 3.42 Å. The outer diameter of B is 116 Å and the average interlayer spacing is 3.47 Å. As is evident to the eye, the inner 22 fringes are more intense than the outer 10 and 12 fringes. About one third of the nanotube length from the actual negative is depicted in Fig. 1.

FIG. 2 Typical scan of fringe intensities of the TEM negative of the two adjacent (nested) nanotubes shown in Fig. 1. Although any one scan has fluctuations in peak intensities, it is clear that the inner fringes are substantially more intense than the outer fringes. The TEM negative is placed in a Jarrel-Ash scanning microphotometer which projects the image onto a slit. The fringes of the double tube are aligned parallel to the microphotometer slit and the negative is scanned and peak intensities recorded on a Linear Instruments chart recorder. The scan rate is 2.5 mm min^{-1} , the slit width is set to 43 microns, and the slit length to 1 mm; the plotted peak-to-peak distance is $\sim 5.5 \text{ mm}$, and the average peak-to-peak distances are determined for tubes A and B by measuring the peak-to-peak separation across 8 peaks (tube A) and 10 peaks (tube B). The 2 outermost peaks are not included for each of A and B in determination of average interlayer (d) spacings. Distances are converted to Å units by calibration against distances obtained from TEM negatives of two standards: encapsulated $\alpha\text{-LaC}_2$ crystals (ref. 25) and, separately, Au particles. The ultimate calibration is against powder X-ray diffraction of the $\alpha\text{-LaC}_2$ particles (ref. 25). We determine the d

determined by the scanning microphotometer, as are the inter-layer (d) separations.

As can be seen from Fig. 2, the peak heights obtained in a single microdensitometer scan are not uniform. Fluctuations in peak height for a particular layer are seen as a function of distance along the long axis of the tube. Also, one might expect the difference in average interlayer separations d_{inner} and d_{outer} to be better determined from several scans across the adjacent tubes. Three scans were obtained and average d values determined by two different methods. The distances obtained are summarized in Table 1, which clearly shows that the average d_{inner} is about 0.08 Å less than the average d_{outer} . Also, it is evident that there is a significant difference between the nested nanotubes A and B, as indicated by the absolute magnitudes of d_{inner} and d_{outer} . That is, it appears that the two nested nanotubes are fundamentally different in their geometry, which could be a result of different helicities. However, the deformation of each is quite similar, as reflected in the $(d_{\text{outer}} - d_{\text{inner}})$ values and peak height patterns obtained.

To compare with an isolated tube, we determined the two average d_{outer} values (each side) for a single (nested) nanotube, and found essentially no difference in the values for several scans. We also scanned the negative of the double tube in reverse order before scanning, and obtained results identical to those already discussed.

The data clearly show that the two nested nanotubes are deformed as a result of their contact. A direct view of this deformation would mean imaging the cross-section perpendicular to the long axis of the adjacent nanotubes of Fig. 1. Although we do not have such an image, we obtained understanding of the deformation from theoretical modelling.

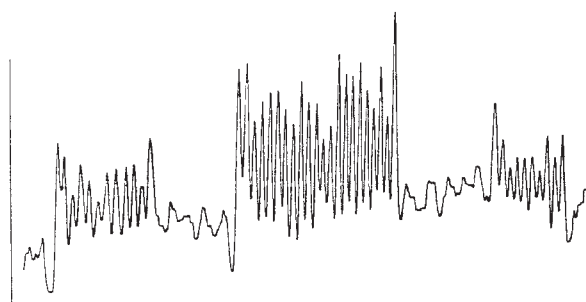
We calculate the structure of a pair of nested carbon nanotubes, using a many-body interaction model for the intralayer forces²³, and an L-J model for the van der Waals interaction between atoms in different sheets, with L-J parameters fitted to graphite²¹. We average all van der Waals interactions in the direction parallel to the cylinder axis. Our nested tubes have an outer diameter of about 50 Å . (Nesting more than two tubes becomes numerically quite difficult.) The atom positions are relaxed until all forces are less than 0.004 eV Å^{-1} .

TABLE 1 Nanotube average interlayer distances*

Scan	A			B		
	d_{outer}	d_{inner}	Δ	d_{outer}	d_{inner}	Δ
1	3.44	3.35	0.09	3.48	3.42	0.06
2	3.46	3.39	0.07	3.51	3.44	0.07
3	3.45	3.39	0.06	3.53	3.43	0.10
Average	3.45	3.38	0.07	3.51	3.43	0.08

Tube A has 10 nested nanotubes, tube B has 12; see Figs 1 and 2.

* All distances in Å. Distances obtained as described for Fig. 2.



spacing of the (200) planes of Au particles to be 2.03 Å with the $\alpha\text{-LaC}_2$ standard; the actual value is 2.04 Å . Absolute distances reported in Table 1 are, therefore, good to 0.5%, and we expect relative distances to be good to 1 part in 500, which is the quoted accuracy of the translation stage of the microphotometer.

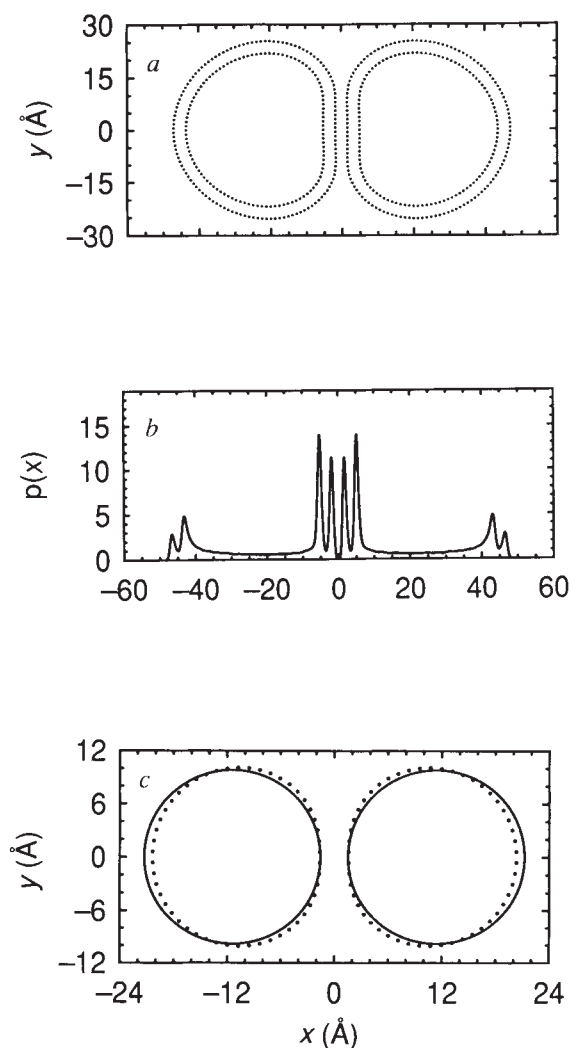


FIG. 3 a, Calculated deformation resulting from van der Waals forces between two (nested) nanotubes. Dots correspond to atom positions. The nanotube diameters are $\sim 50 \text{ Å}$. Interlayer separations after deformation from the (nested) tube/(nested) tube van der Waals interaction are 3.44 Å (outer) and $3.39, 3.37, 3.39 \text{ Å}$ (inner). b, Projected atom density from a. Note that the flattening evident in a is indicated by the larger projected atom density of the inner nanotube layers. c, Calculations for single-layer nanotubes 20 Å in diameter. The undeformed tubes are indicated by solid circles, and the relaxed tubes by dotted lines. The shift in the outer portion of the tubes is large enough (about 1.5 Å) to be observable experimentally.

Our individual tubes are generated by rolling a graphite sheet perpendicular to the 'a' axis, so the helical pitch is zero. Actually, the helicity is virtually irrelevant to the deformation. In the limit that the interlayer separation and tube radius of curvature are much larger than the intra-layer bond length, the tubes act as uniform elastic sheets. These sheets are isotropic in two dimensions because of the underlying hexagonal symmetry. Thus all of the properties discussed here are actually controlled by the planar-averaged van der Waals interaction, and the elastic constant for bending the graphite sheet, neither of which depends on helicity. Because the interlayer spacing is only a factor of 2.41 larger than the in-plane bondlength, small corrections could occur if two sheets had ordered stacking, like graphite. But this is not included in the calculation (because of our averaging of the interaction along the axis), and is unlikely to occur in reality (because sequential tubes may have different helicities, and so may not have graphite-like ordering; see ref. 24).

The resulting structure is shown in Fig. 3a. The deformation is evident: the nanotubes flatten where they are pressed together by the van der Waals interaction. This provides a compelling explanation for the contrast differences between the inner and outer layers of the high-resolution image shown in Fig. 1. The flattening of the inner planes leads to a locally thicker region of planes that satisfy the Bragg condition for diffraction. The resulting enhancement of contrast is illustrated in Fig. 3b.

As in the experiment, the calculation also shows a reduction in the interlayer spacing where the nanotubes are pressed together. Whereas the interlayer spacing at the far left and right of Fig. 3a is 3.44 Å, in the centre the respective spacings are 3.39, 3.37 and 3.39 Å. Thus the inner spacings are reduced by ~0.06 Å relative to the outer spacing, in good agreement with the experimental data in Table 1.

Nanotubes as small as 1 nm in diameter have been reported, and it is to be expected that the deformation will decrease with tube diameter. We have also performed calculations for single-layer tubes with smaller diameters, and find that measurable deformations remain at least for tubes as small as 20 Å in diameter (Fig. 3c). We have also investigated the influence

of nesting by comparing the calculations for nested, two-layer tubes of 50- and 30-Å diameter with those for single-layer tubes. Nesting causes a moderate reduction in the extent of deformation.

We anticipate that van der Waals forces will be equally important in determining the structure of nanotubes bound to substrates, or into bundles, and that in all of these situations the intermolecular forces will play an important part in determining nanotube geometry and properties. Thus it seems unlikely that nanotubes are ever truly cylindrical. □

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Synthesis of a tubular polymer from threaded cyclodextrins

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MUCH attention has been focused recently on the design and fabrication of large-scale molecular structures¹. Carbon nanotubes formed by an arc-discharge method^{2,3} have attracted particular attention. These tubes range from about 1 to 30 nanometres in diameter and a micrometre or so in length. To construct smaller tubes, direct chemical synthesis may be a more convenient approach. The cyclic oligomers of glucose known as cyclodextrins (CDs) would seem to be ideal candidates for the components of a molecular tube: they contain cylindrical cavities about 0.7 nm deep, with diameters of 0.45 nm, 0.7 nm and 0.85 nm for α -CD, β -CD and γ -CD respectively⁴. Lehn has recently reported the use of 'bouquet molecules' built from β -CD with long side chains as artificial ion channels⁵. We have previously prepared rotaxane supermolecules in which CDs are threaded on a polymer chain, and we⁶ and others^{7,8}, have reported polyrotaxanes with many threaded CDs. Here we report the crosslinking of adjacent CD units in a

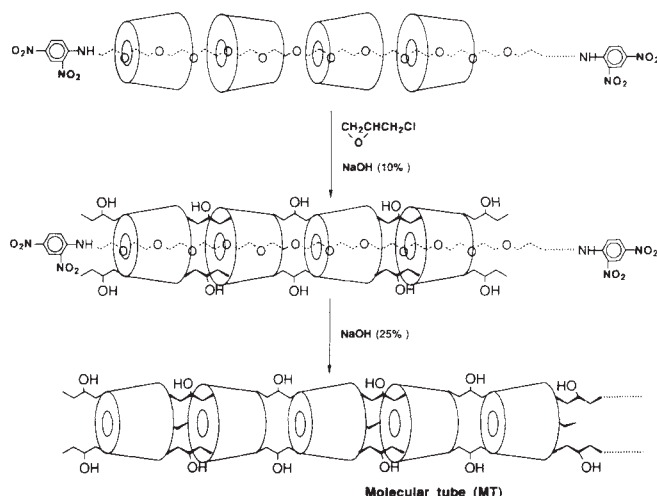


FIG. 1 Preparation of a molecular tube (MT) from polyrotaxane (MN).

polyrotaxane to create a molecular tube. By removing the bulky ends of the polymer thread, the tube can be unthreaded and can act as a host for reversible binding of small molecules.

Polyrotaxanes were prepared as described⁶. In this case poly(ethylene glycol) (PEG) of molecular mass 1,450 was used, because α -CD forms complexes with PEG of molecular weight 600–2,000 most efficiently. The complexes are nearly stoichiometric.

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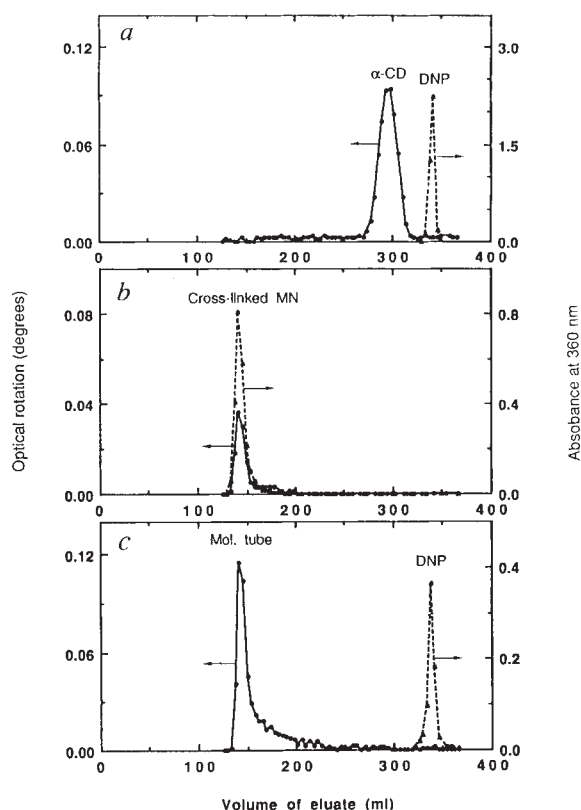


FIG. 2 Elution diagrams of the reaction mixture of polyrotaxane with 25% NaOH (a), that of the crosslinked polyrotaxane (MN) (b), and that of the reaction mixture of the crosslinked polyrotaxane with 25% NaOH (c). A Sephadex G-25 column (2.2 $\times$ 93 cm) with water eluant was used. Absorbance was measured at 360 nm (with cell length 1 cm) and optical rotation was measured at 589 nm (with cell length 10 cm).

metric (two ethylene glycol units : one CD), which means that α -CDs are almost close-packed from end to end of the polymer chain. The polyrotaxane (22.5 mg) (shown as MN in the reaction scheme, Fig. 1) was dissolved in a solution of 10% NaOH, to which epichlorohydrin (3.84 mmol) was then added. The solu-

tion was stirred at room temperature for 36 hours and then neutralized by the addition of HCl. The resultant yellow solid was purified by precipitation from ethanol. In order to remove the bulky stoppers ($\text{NH}(\text{C}_6\text{H}_3)(\text{NO}_2)_2$ groups) at each end of the molecules, the product was treated with a strong base (25% NaOH) at 45 $^\circ\text{C}$ for 24 hours. The reaction mixture was cooled and then neutralized with HCl. Figure 2 shows the elution diagrams of a reaction mixture of polyrotaxane with 25% NaOH (Fig. 2a), of the crosslinked polyrotaxane (Fig. 2b), and of the reaction mixture of the crosslinked polyrotaxane with 25% NaOH (Fig. 2c). The crosslinked product was eluted at the void volume (Fig. 2b), the same as the polyrotaxane. After the polyrotaxane was treated with 25% NaOH and the solution was neutralized before crosslinking, two peaks were observed (Fig. 2a): one could be detected only by absorbance in the ultraviolet (at 360 nm), which is assigned to the dinitrophenyl group (DNP), and the other, which could be detected only by optical rotation, is identified as dethreaded α -CDs. After the crosslinked product was treated with strong base, two peaks were observed (Fig. 2c): the one at the void volume, which is detected only by optical rotation, is identified as the product, the molecular tube. The second peak, that was detected only by absorbance in the ultraviolet (at 360 nm), is assigned as a dinitrophenyl group (DNP). Figure 3 shows the elution diagram of the molecular tube (Fig. 3a) on Sephadex G-100 (the exclusion limit is 4,000–100,000) together with those (Fig. 2b) of dextran (M_r 20,000) and α -CD. The molecular tube was eluted just after dextran (M_r 20,000), indicating that the average molecular mass of the molecular tube

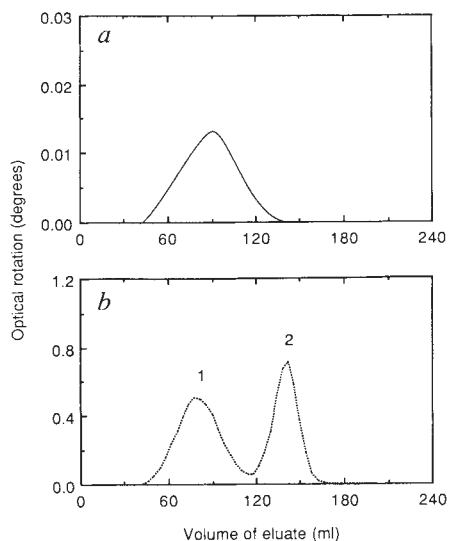


FIG. 3. Elution diagram of (a) the molecular tube and (b) dextran (peak 1) (M_r 20,000) and α -CD (peak 2). A Sephadex G-100 column (1.8 $\times$ 55 cm) with water eluant was used.

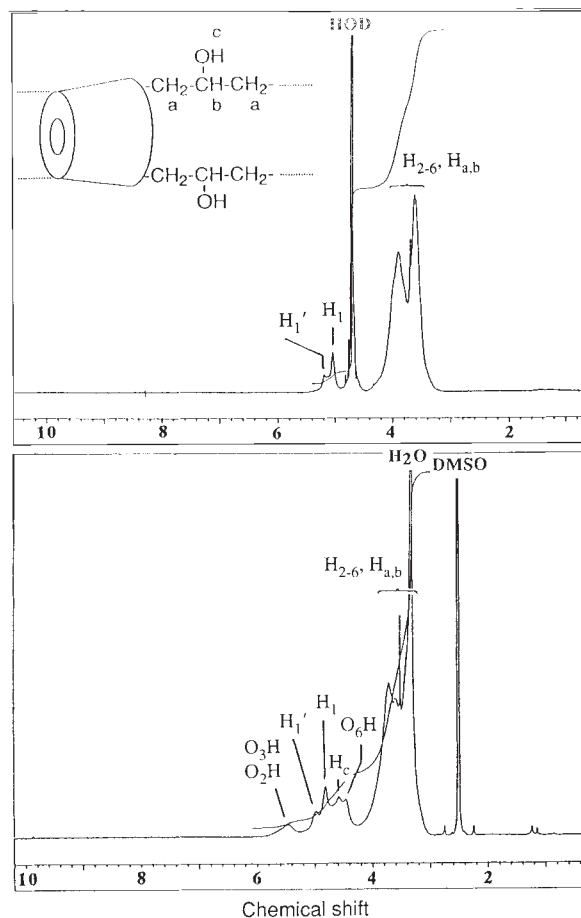


FIG. 4 ^1H NMR spectra of the molecular tube in D_2O (upper) and in dimethyl sulphoxide- d_6 (lower). H_1 shows the H_1 proton having a crosslinking bridge. Chemical shifts are given in p.p.m. relative to solvent (DMSO, 2.5 p.p.m., and H_2O , 4.7 p.p.m.).

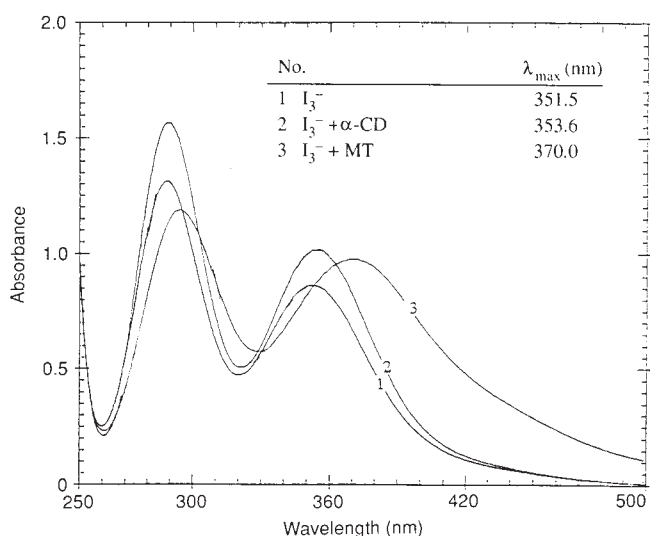


FIG. 5 The absorption spectra of $KI-I_2$ solutions in the absence (1) and presence (2) of α -CD (0.4 g l^{-1}) and presence (3) of molecular tube (MT) (0.4 g l^{-1}). $[I_3^-] = 2.0 \times 10^{-4} \text{ M}$. Cell length is 1 cm.

is less than 20,000. This value is consistent with the fact that the molecular mass of the molecular tube, prepared from PEG of molecular mass 1,450, is $\sim 17,000$. Therefore, intrachain crosslinking took place predominantly. The yield of the final product is 92%. Its elemental analysis gave the following results: found (calculated with three crosslinking bridges between CDs for $C_{45}H_{72}O_{33}(H_2O)_2$): C, 45.88 (45.92); H, 6.67 (6.51).

The product was soluble in water, dimethylformamide (DMF), and dimethylsulphoxide (DMSO), although polyrotaxanes are insoluble in water and DMF, and soluble in DMSO.

The product was characterized by 1H NMR, ^{13}C NMR, infrared and ultraviolet spectroscopy, and gel permeation chromatography. Figure 4 shows the 1H NMR spectra of the molecular tube in D_2O and in $DMSO-d_6$. The 1H NMR and ^{13}C NMR spectra show that CD, the bridge, and the H_1 proton with the bridge can be observed. All the peaks of the 1H NMR spectrum are broadened, indicating the product is polymeric.

When the solution of the molecular tube was added to a pale yellow solution of potassium iodide containing free iodine ($KI-I_2$), a deep red colour was instantaneously produced, although the addition of an α -CD solution to $KI-I_2$ solution caused no colour change. Figure 5 shows the absorption spectra of $KI-I_2$ solution in the presence and absence of α -CD and molecular tube. On addition of α -CD, the position of the absorption maximum changed little, with some increase in intensity. On addition of the molecular tube solution, the absorption maximum shifted to longer wavelength and a large tailing over 500 nm was observed. On addition of randomly crosslinked α -CD with epichlorohydrin, no visible changes took place. These results indicate that I_3^- ions are arranged linearly in the tube. The shift is not so large compared with that observed with amylose and $KI-I_2$ solution, but is similar to that observed with amylopectin. The change in the spectrum was found to be a maximum at a ratio of CD unit to I_3^- molecule of 1:1. These findings indicate that such template synthesis provides an approach to the construction of new nanostructures. □

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Annual growth banding in a cave stalagmite

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THE presence of microscopic luminescence banding in cave calcite deposits (speleothems) has recently been reported^{1–3}. The luminescence seems to be caused by excitation of humic and fulvic acids derived from the overlying soil and subsequently incorporated into the calcite^{3,4}. We have tested whether such luminescence corresponds to annual growth layers, using high-precision thermal-ionization mass-spectrometric ^{238}U – ^{234}U – ^{230}Th ages from banded sections of a Holocene stalagmite. We report here that the timespans yielded by counting bands, on the assumption that they are annual, agree within error limits with those obtained by our uranium–thorium dating. This demonstration that the banding is annual should make banded speleothems valuable resources for providing high-resolution records of past climate (in which precipitation can be estimated from growth rates^{5,6} and temperatures from stable-isotope measurements⁷) and solar–terrestrial correlations (as the luminescence intensity can be related to the solar sunspot cycle^{1–3}).

Forty-three speleothem samples were collected from karst areas in the United Kingdom. After cutting along the axis of growth, 2-mm-thick polished sections were prepared. These were

observed under a standard Zeiss microscope fitted with an IV Fl epi-fluorescence condenser containing a 50 W super-pressure mercury lamp. A 320–420 nm excitation filter was used to transmit the portion of ultraviolet light known to generate a strong luminescence from organic matter (around 350–380 nm; ref. 4), and the luminescence itself viewed using a 420-nm barrier filter, which prevented transmission of the excitation waveband. A magnification of between $\times 64$ and 100 was used to observe luminescence, which was recorded using long-exposure photography on high-contrast Kodak Ortho 6556 (type 3) film. This was then scanned to give a digitized image. A 5 pixel (125 μm) smoothing filter was then applied to remove noise, permit clear separation of consecutive bands, and allow measurement of peak to peak bandwidth. Assuming that the distance between successive peaks represents the annual growth of the sample, the average axial growth rate can then be determined.

Banding occurred only in a few samples (5 out of 43 examined). This may be due to the use of a mercury lamp source, which has a low-energy broad spectrum output, and may not be powerful enough to excite weak luminescence banding. Although Shopov *et al.* used an ultraviolet laser source, they also noted that annual banding was apparently absent in some samples². The limited preservation of annual banding may be related to strict requirements on the type of percolation flow feeding the speleothem; soil water and unsaturated zone storage need to be adequate to maintain flow during the summer, while residence times overall remain sufficiently short that summer and winter waters are not completely mixed. Crystal structure and orientation also appear to be important factors in signal preservation, but further investigation is needed. Finally, it would also be useful to compare the luminescence record with that of visible banding, which has occasionally also been reported in speleothem^{8,9}.

TABLE 1 Uranium/thorium isotopic data and mass-spectrometric ages for SU-80-11

Sample name*	Distance down axis*	^{238}U concentration ng g $^{-1}$	$^{234}\text{U}/^{238}\text{U}$ $\times 10^6$	$^{230}\text{Th}/^{232}\text{Th}$ $\times 10^{-6}$	$\delta^{234}\text{U}(\text{O})^{\dagger\dagger}$ ‰	$\delta^{234}\text{U}(\text{T})^{\dagger\dagger}$ ‰	$[^{230}\text{Th}/^{238}\text{U}]_{\text{act}}^{\S\dagger}$	Age ‡ years BP	Corrected age ¶ years BP
SU-80-11A	2 mm	247.61 $\pm$ 0.75	64.64 $\pm$ 0.36	26.3 $\pm$ 0.9	181.2 $\pm$ 6.6	185.7 $\pm$ 6.8	0.0878 $\pm$ 0.0029	8,340 $\pm$ 290	6,970 $\pm$ 750
SU-80-11B	28 mm	254.76 $\pm$ 0.75	64.38 $\pm$ 0.43	110.1 $\pm$ 3.0	176.5 $\pm$ 7.8	179.0 $\pm$ 7.9	0.0506 $\pm$ 0.0014	4,730 $\pm$ 130	4,540 $\pm$ 160
SU-80-11C	64 mm	237.11 $\pm$ 0.48	66.84 $\pm$ 0.27	148.7 $\pm$ 4.5	221.4 $\pm$ 4.9	223.6 $\pm$ 5.0	0.0378 $\pm$ 0.0011	3,370 $\pm$ 100	3,270 $\pm$ 110
SU-80-11D(1)	80 mm	329.49 $\pm$ 0.30	65.97 $\pm$ 0.12	149.9 $\pm$ 2.3	205.6 $\pm$ 2.1	207.4 $\pm$ 2.2	0.0339 $\pm$ 0.0005	3,103 $\pm$ 43	3,014 $\pm$ 62
SU-80-11D(2)		329.56 $\pm$ 0.24	65.90 $\pm$ 0.11	147.0 $\pm$ 6.5	204.4 $\pm$ 2.1	206.2 $\pm$ 2.1	0.0333 $\pm$ 0.0014	3,051 $\pm$ 131	2,962 $\pm$ 138
SU-80-11E	96 mm	302.95 $\pm$ 1.11	67.31 $\pm$ 0.31	1622 $\pm$ 95	230.1 $\pm$ 5.6	231.9 $\pm$ 5.7	0.0304 $\pm$ 0.0008	2,680 $\pm$ 70	2,670 $\pm$ 70

* Sample location and distances shown in Fig. 1; SU-80-11D(1) and SU-80-11D(2) are replicate analyses of different aliquots of the solution derived from sample SU-80-11D.

$\dagger \delta^{234}\text{U} = ([^{234}\text{U}/^{238}\text{U}]/([^{234}\text{U}/^{238}\text{U}]_{\text{eq}}) - 1) \times 10^3$, where $(^{234}\text{U}/^{238}\text{U})_{\text{eq}}$ is the atomic ratio at secular equilibrium and is equal to 5.472×10^{-5} . $\delta^{234}\text{U}(\text{O})$ is the measured value. $\delta^{234}\text{U}(\text{T})$ is the initial value and is equal to $\delta^{234}\text{U}(\text{O})e^{\lambda_{234}T}$.

$\ddagger$ Values for decay constants are $\lambda_{238} = 1.551 \times 10^{-10} \text{ yr}^{-1}$ (ref 15), $\lambda_{234} = 2.835 \times 10^{-6} \text{ yr}^{-1}$ (refs 16, 17), and $\lambda_{230} = 9.195 \times 10^{-6} \text{ yr}^{-1}$ (ref 18).

$\S$ Calculated from the atomic $^{230}\text{Th}/^{238}\text{U}$ ratio by multiplying by $\lambda_{230}/\lambda_{238}$.

$\parallel$ Ages are calculated using $[^{230}\text{Th}/^{238}\text{U}]_{\text{act}} - 1 = e^{-\lambda_{230}T} + (\delta^{234}\text{U}(\text{O})/1000)(\lambda_{230}/\lambda_{234})(1 - e^{-(\lambda_{230} - \lambda_{234})T})$ where T is the age in years.

$\P$ Corrected ages assume an initial $^{230}\text{Th}/^{232}\text{Th}$ atomic ratio of $4.4 \pm 2.2 \times 10^{-6}$ (method 1, equation 8 from ref. 19). This is the value for a material at secular equilibrium, with a crustal $^{232}\text{Th}/^{238}\text{U}$ value of 3.8. The error is arbitrarily assumed to be 50%. The error in the corrected age for SU-80-11A is large because of detrital contamination and the uncertainty in the initial $^{230}\text{Th}/^{232}\text{Th}$ ratio used in correcting for this contamination.

The Holocene stalagmite SU-80-11, collected from Uamh an Tartair Cave, Sutherland (NW Scotland), was found to contain dense luminescence banding at irregular intervals along its length, an example of which is shown in Fig. 1. This banding was not continuously preserved down the section, with the longest unbroken record covering 36 years. Figure 2 presents the complete record of bandwidths obtainable along the growth axis in the form of the mean and standard deviation of sets of five consecutive bands. Along the two portions of the sample for which luminescent bands were visible (1 and 2 on Fig. 2), the growth rates are $0.0549 \pm 0.0027 \text{ mm yr}^{-1}$ and $0.0565 \pm 0.0029 \text{ mm yr}^{-1}$ (2 s.e.m.; number of bands, $n=242$ and 66, respectively). The mean growth rates do not vary significantly for the two intervals, whereas inter-annual variability is of a magnitude comparable to that of present annual precipitation variations (work in progress).

The development of high-precision thermal-ionization mass-spectrometric ^{238}U – ^{234}U – ^{230}Th (TIMS ^{230}Th) dating^{10–12} and its application to speleothems¹³ provides a precise means of testing the growth rate of the intervals with luminescence banding. Using a diamond wire saw, five sub-samples (A–E) of less than 2 g weight were cut from SU-80-11 for TIMS ^{230}Th dating (Fig. 1). Analytical methods are described in refs 10–12, but modified for use on a Finnigan-MAT 262-RPQ mass spectrometer, which is optimized for measuring small ion beams. Ion counting techniques with low dark noise combined with high-abundance sensitivity allowed SU-80-11 to be dated despite low levels of ^{230}Th (380 to 960 million atoms per g of speleothem) and relatively low $^{230}\text{Th}/^{232}\text{Th}$ (26.3 to $1,622 \times 10^{-6}$). ^{230}Th ion currents, measured on the multiplier before the (unused) quadrupole

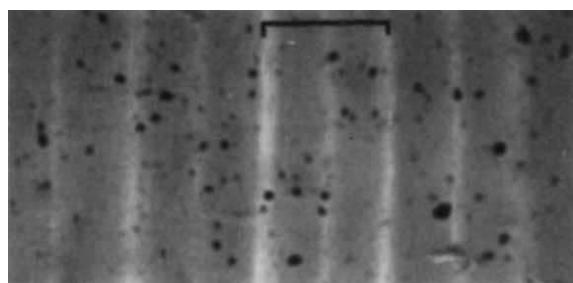


FIG. 1 Photomicrograph of luminescent banding 85 mm from base of sample (approximate age, $2,950 \pm 150$ years). Nine consecutive bands are visible under microscope magnification of $\times 100$. Black spots are dust particles entrapped within the UV apparatus, and are not a feature of the speleothem. Scale bar, 0.1 mm.

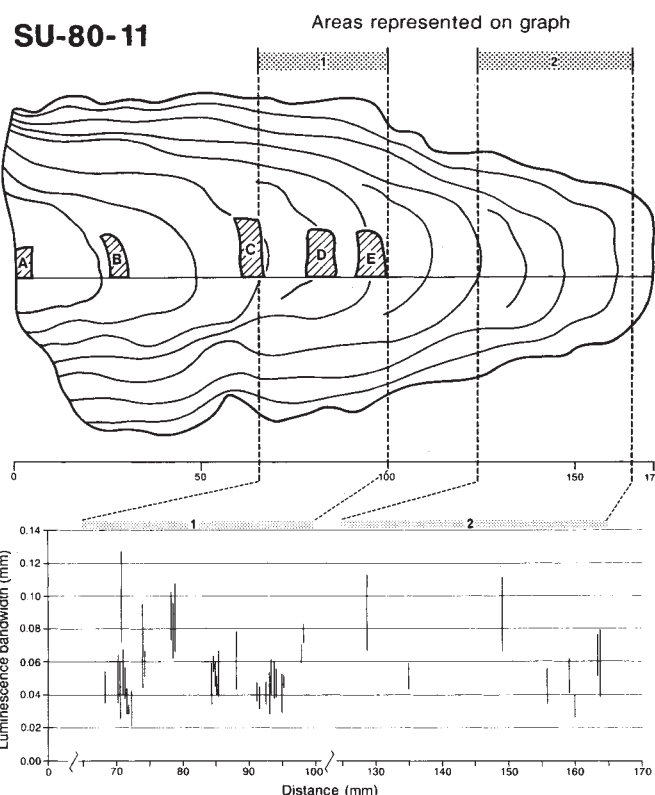


FIG. 2 SU-80-11, showing position of samples for TIMS ^{230}Th dating and luminescence bandwidth record. Bandwidth equals growth rate (mm yr^{-1}) if banding is annual. Because of the large number of bands observed, data is only presented where five consecutive luminescence bands occur; the length of each bar representing $\pm 1\sigma$ of the population of the five-band set, and the centre the arithmetic mean.

second stage, ranged from 10 to 20 c.p.s.; 3,000 to 9,000 ^{230}Th ions were counted in each mass spectrometer run. Within-run statistics were comparable to counting statistics. The TIMS ^{230}Th ages obtained are listed in Table 1. Replicate analyses agree within error indicating that the analyses are reproducible. The ages are also consistent with their stratigraphic positions within the speleothem.

The sample grew during a period when climatic conditions changed relatively little in the UK (the late climatic optimum)¹⁴. Deposition appears to be continuous in SU-80-11 with no growth hiatuses visible, thus absolute growth rates can be calculated between sub-samples. There is a general increase in growth

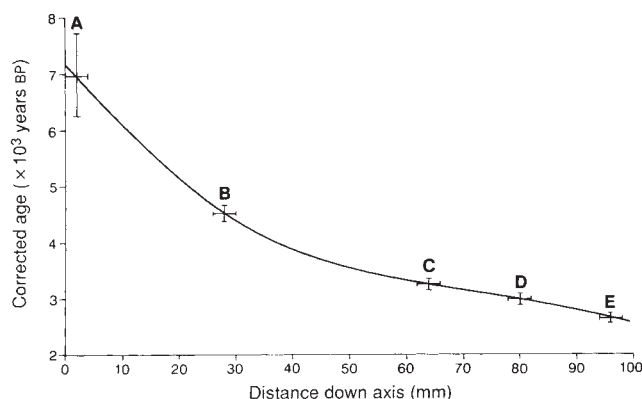


FIG. 3 Graph of TMS ^{230}Th ages against distance up the growth axis of SU-80-11. The gradient of the curve is equivalent to the growth rate, and shows a slow decrease in growth rate with time.

rate over time, but between samples C and E (interval 1), growth rate is constant (Fig. 3). These samples span a 32-mm section of the stalagmite for which the luminescence record also allows determination of a growth rate (assuming annual banding) of $0.0549 \pm 0.0027 \text{ mm yr}^{-1}$ (2 s.e.m.; $n=242$). This gives an age difference between sub-samples C and E of 586 ± 28 years, which compares with a difference of 600 ± 130 years derived from the TMS ^{230}Th ages (Table 1 and Fig. 3). The difference between these is well within the 2σ analytical uncertainty associated with the two estimates, and it can be concluded that the luminescence banding is indeed annual in nature.

Luminescence banding in speleothems, here demonstrated for the first time by TMS ^{230}Th dating to be annual, can be used to provide an annual signal for high-resolution palaeoclimatic studies. First, as suggested by previous workers³, annual variations of intensity of the luminescence may provide information on changes in solar output. They demonstrated that one flow-stone sample showed peaks in luminescence intensity with a periodicity of 0.95, 8.6, 10.4 and 13.3 bandwidths. These were thought to be caused by variations in plant productivity associated with both the annual temperature cycle (0.95 bandwidth) and the 11-year sun-spot cycle (8.6, 10.4 and 13.3 bandwidths). Second, the distance between bands can be used to provide measures of growth rate variation. As shown by this sample, significant variations in growth rate occur, and may be influenced by both temperature and precipitation, as theoretically modelled by Dreybrodt^{5,6}. The potential of this palaeoclimate signal is currently under assessment, and may provide a unique proxy record of palaeoprecipitation. Furthermore, the banding itself can be used as a high resolution chronostratigraphic tie between TMS ^{230}Th ages, and other high-resolution records obtainable from speleothems (trace elements, oxygen and carbon isotopes)⁷. □

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Blueschist metamorphism in an active subduction zone

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THE high-pressure, low-temperature metamorphic rocks known as blueschists have long been considered to form in subduction zones, where the descent of a relatively cold slab leads to the occurrence of unusually low temperatures at mantle pressures. Until now, however, the link between blueschist-facies rocks and subduction zones has been indirect, relying on a spatial association of blueschists with old subduction complexes, and estimates of the geothermal gradients likely to exist in subduction zones. Here we strengthen this link, by reporting the discovery of blueschist-facies minerals (lawsonite, aragonite, sodic pyroxene and blue amphibole) in clasts from a serpentinite seamount in the forearc of the active Mariana subduction zone. The metamorphic conditions estimated from the mineral compositions are $150\text{--}250^\circ\text{C}$ and $5\text{--}6 \text{ kbar}$ ($16\text{--}20 \text{ km}$ depth). The rocks must have been entrained in rising serpentine mud diapirs, and extruded from mud volcanoes onto the sea floor. Further study of these rocks may provide new insight into the tectonics of trench-forearc systems, and in particular, the processes by which blueschist-facies clasts come to be associated with forearc sediments in ancient subduction complexes.

During Ocean Drilling Program Leg 125, scientists drilled two serpentinite seamounts in the Mariana and Izu-Bonin forearcs¹. They recovered abundant serpentinized peridotites and minor amounts of low-grade metamorphosed mafic rocks from both seamounts. The mineral assemblages and chemistries show that a bimodal pressure assemblage is represented within these low-grade metamorphic rocks: one at low, and one at high pressure². The high-pressure assemblage, containing blueschist-facies minerals, was present only in one hole at ODP Site 778 in the Mariana forearc.

Hole 778A was drilled to a depth of 107.6 metres below the sea floor (m.b.s.f.) on the southern flank of Conical Seamount. This seamount is located in the Mariana forearc, $\sim 80 \text{ km}$ west of the trench axis (Fig. 1). The drill core contained ~ 60 metamorphosed mafic rocks as subrounded pebble-size clasts in a serpentine matrix. These protoliths were mainly aphyric to fine-grained basalt and amphibolite, although later-stage cataclastic deformation and chloritization usually obliterate the primary textures and obscure their origin.

Nine specimens of different lithologies from Hole 778A were

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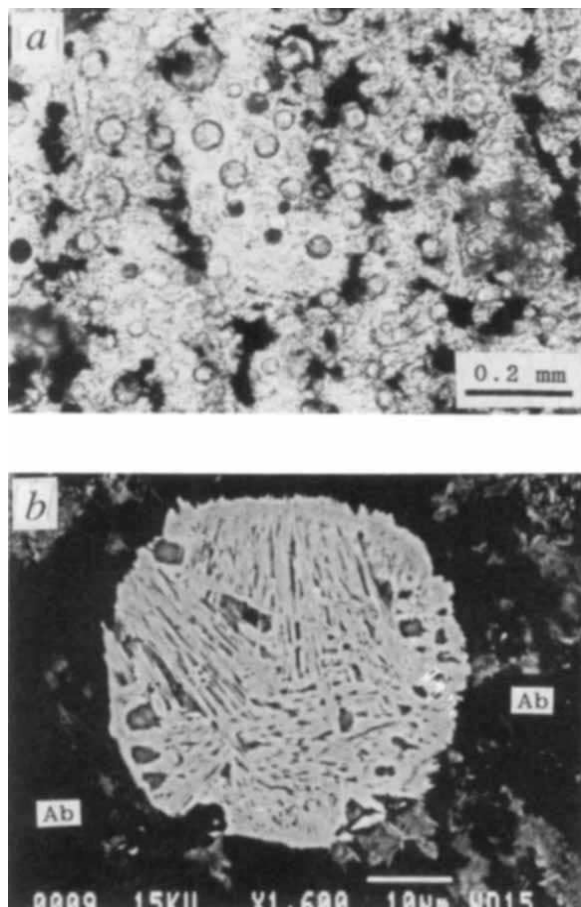


FIG. 2 Microphotograph (a) and back scattered image (b) showing spherulitic aggregates of acicular sodic pyroxene in albite. Ab, albite. Scale bar in b is 10 µm.

aggregates of acicular crystals in plagioclase phenocrysts pseudomorphosed by albite, (2) irregularly shaped aggregates of acicular crystals in pumpellyite, and (3) epitaxial fringes along the cleavage traces of igneous clinopyroxene (Fig. 2). Its presence was confirmed by X-ray diffraction. Sodic pyroxene usually coexists with albite, pumpellyite, chlorite and quartz. Most analysed sodic pyroxenes in our samples are scattered around the chloromelanite field and contain 20–40% jadeite component.

The blue amphibole-bearing specimen (13R, CC piece no. 4, 6–10 cm) is characterized by fabrics that suggest strong tectonic shearing, and contains microclasts of clinopyroxenite, serpentinite, and amphibolite in a foliated chlorite and clay matrix. The tectonic fabrics of the specimen suggest that the rock is a type of fault gouge and may have been formed as a result of accumulation of materials which were torn from sheared blocks of mafic to ultramafic rocks during rise of the serpentinite mass in which it is entrained. The size of the microclasts is variable, from <0.2 mm to > 8 mm in length. Boudinage structures of microclasts and pressure shadows of chlorite around porphyroclasts are common. Blue amphibole (with distinct pleochroism from blue to pale violet) occurs along the rims of cleavage traces of relict hornblende, and within pulled-apart fractures of boudinaged relict hornblende. This suggests syntectonic recrystallization. Microprobe analyses indicate that the later amphibole is a solid solution between actinolite and magnesioriebeckite, and can be classified as winchite in the sense of Leake⁶. This kind of winchite favours high pressure conditions⁷.

The approximate conditions of metamorphism can be estimated using published results of theoretical and experimental inves-

tigations. The presence of aragonite, rather than calcite, provides evidence that the pressure lay above the aragonite-calcite inversion curve (>4.5 kb at 150 °C and >6 kb at 200 °C)⁸. The jadeite component of the sodic pyroxene, which is accompanied by quartz and albite, is about 30 mol %. Using calibrated isopleths for the jadeite component, we estimate pressures of 5 kb at 150 °C and 6 kb at 250 °C. Unusual textures and significant range of jadeite components of sodic pyroxene, however, may be due to kinetics rather than equilibrium recrystallization. They may have been caused by metamorphism at a higher pressure, produced by high pressure overstepping of the equilibrium reaction curve. The reaction heulandite = lawsonite + quartz + fluid⁹ gives a minimum temperature of 200 °C for the lawsonite + quartz association, and the reaction lawsonite + pumpellyite = zoisite + chlorite + quartz + fluid gives a maximum temperature of 250 °C for the lawsonite + pumpellyite association¹⁰. The Fe₂O₃ content of lawsonite probably affects these stability limits. Liou *et al.*¹⁰ demonstrated that the introduction of Fe₂O₃ in the latter reaction shifts the reaction curve to a lower temperature. Thus, the metamorphic conditions under which our blueschist-facies samples formed were roughly 150–250 °C and 5–6 kb (16–20 km depth).

Since Eocene time (~45 million years ago), the Pacific plate has been subducting beneath the Philippine Sea plate along the Mariana trench¹¹. The mantle peridotite above the subducting slab has reacted with water to form serpentinite, and the resulting low density material has risen (perhaps aided by faulting in the forearcs) to form a zone of seamounts in the forearc area between 50 and 150 km from the trench axis¹². The Conical Seamount, situated ~30 km above the subducting slab, is one of the typical diapir seamounts and the low density fluids (which converted peridotite to serpentinite) are still actively upwelling from its summit¹³. It is hard to see how the blueschist-facies materials could have been derived from any source other than the Mariana subduction system, because the system is much too far away from any continental margin where blueschist belts are exposed. Therefore, we have inferred that the blueschist-facies

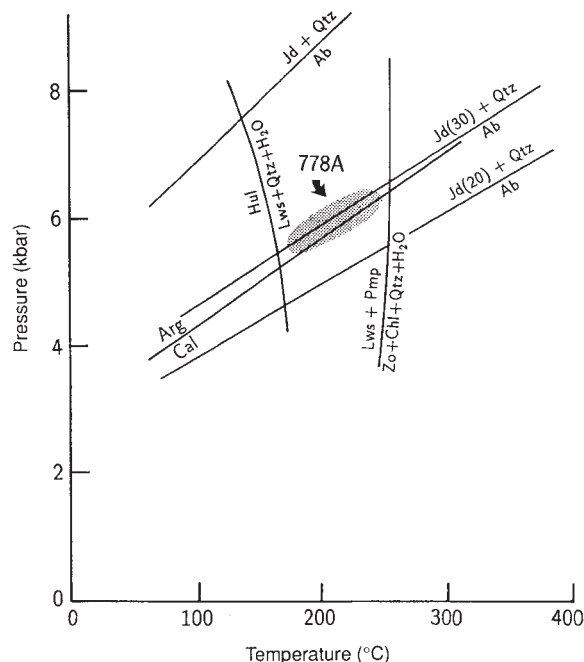


FIG. 3 Approximate metamorphic conditions of the rocks from Hole 778A. Pressure-temperature curves for some pertinent metamorphic relations are also shown in this figure^{8–10}. Arg, aragonite; Cal, calcite; Hul, heulandite; Lws, lawsonite; Qtz, quartz; Jd, Jadeite; Ab, albite; Pmp, pumpellyite; Zo, zoisite; Chl, chlorite.

rocks recovered from Hole 778A formed after the initiation of the subduction, at about 16–20 km below the seamount. Thus, the serpentine mud in which the blueschist-facies clasts are entrained could have initiated at least from that depth. Synmetamorphic cataclastic deformation is ubiquitous in these clasts. Such brittle deformation is consistent with a low temperature of metamorphism within the subduction zone.

During the last two decades, most blueschist-facies metamorphic events have been surmised to occur in and around subduction zones^{14–17}. The discovery of blueschist-facies clasts in the seamounts described above provides direct evidence that blueschist-facies metamorphism really takes place in association with the subduction process. Numerous tectonic models to explain uplift processes of high P/T blocks have been proposed, mainly for the Franciscan terrane^{18–22}. The models of occurrence of blueschist-facies clasts in serpentinite seamounts in the modern Mariana forearc system fit models which attribute the uplift of blueschists to serpentinite diapirism in forearc regions^{18,22}.

Although the problems of uplift of blueschist terranes are beyond the scope of this paper, we may conclude that serpentinite diapirism is one of the potentially important mechanisms for uplifting blueschist-facies blocks from subduction zones to forearc surfaces. This mechanism may have caused the recycling of blueschists as observed in the Franciscan complex^{23,24} and in the Kamuikotan blueschist-facies terranes of Japan^{25,26}. It can also explain the occurrence of clastics of serpentinite and blueschist in the Great Valley Sequence^{22,27} and in the Yezo Group^{28,29}, which are recognized as forearc basin sediments during the Franciscan and the Kamuikotan metamorphic events, respectively.

Although we obtained only incipient blueschist-facies rocks from ODP Leg 125, there are probably higher-grade blueschists and eclogites yet to be found in Conical Seamount and other serpentinite seamounts in the Mariana forearc. Further reconnaissance is necessary to clarify physico-chemical conditions and dynamics at deeper parts of the subduction zone beneath the Mariana forearc. □

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Daytime calibration of magnetic orientation in a migratory bird requires a view of skylight polarization

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THE orientation of migratory birds is based on a complex of interacting compass mechanisms (the geomagnetic field, stars, patterns of skylight polarization and, perhaps, the Sun)^{1,2}. A magnetic compass develops in birds that have never seen the sky^{3–8}, but the preferred direction of magnetic orientation may be modified during the first three months of life by exposing naive birds to either the clear daytime or night sky under conditions in which magnetic directions differ substantially from true or geographic directions^{5–7,9}. We hypothesized that celestial rotation, which indicates geographic directions both day and night, served as the calibrating reference⁷, and showed that a rotating pattern of artificial stars provided a sufficient stimulus to calibrate magnetic orientation in young Savannah sparrows (*Passerculus sandwichensis*)¹⁰. During daytime either the Sun's disc or patterns of polarized skylight could provide the reference to geographic compass directions^{11,12}. Here we report that visual access to natural skylight polarization patterns is necessary for calibration of magnetic orientation during daylight.

The Savannah sparrow is a medium-distance nocturnal migrant that nests in grassland, meadow and tundra across North America from the northern United States northwards to 60–70° N. It migrates to the southern United States southward to northern Central America in winter. Adult Savannah sparrows possess magnetic^{8,13} and star compasses⁵, and use visual cues associated with clear sunset skies (including skylight polarization patterns)^{14–16} for orientation. To study the ontogeny of magnetic orientation, we transferred nestling sparrows from nests in the field to a laboratory incubator before their eyes opened (2–3 days post-hatching) and hand-raised them to independence. Until 50–70 days old the birds lived in a normal magnetic field in the ambient photoperiod, but had no view of the outdoors or sky. Sixty-three birds from 19 nests were each assigned to one of four groups such that nest mates were, to the greatest extent possible, placed in different groups. On sunny days between 19 August and 23 September, 1992, birds were placed outdoors in Emlen funnel cages¹⁷ from which they could view the sky. The cages were placed within a large (2.7 m) Rubens coil¹⁸ that was used to shift the magnetic field direction. Birds were given exposure to the sky, 3–4 h at a time, throughout the day from sunrise to sunset (each group experienced 25–28 h of exposure over 8–9 days). The experience of the four groups differed with respect to whether they could see skylight polarization patterns and whether they viewed the sky in a shifted or unshifted magnetic field. The four treatment groups were: (1) birds that could view the clear sky through black nylon crop netting (2-cm mesh), within the normal geomagnetic field (5.5×10^4 nT; inclination = 70°; declination = 13° W); (2) birds exposed to the natural sky within a magnetic field shifted 90° counterclockwise (CCW) (5.5×10^4 nT; inclination = 69°; mag-

FIG. 1 Magnetic orientation of the four groups of birds tested indoors in orientation cages covered with white translucent plastic sheets. Birds were tested in the unshifted geomagnetic field (●) and in a field in which magnetic north was shifted 90° CCW (○). Because the directions chosen were the same under the two field conditions, the data were pooled for statistical analysis with a single datum included for each bird. *a*, Orientation of the birds (group 1) exposed to the natural clear daytime sky in an unshifted magnetic field. The group showed NNW-SSE magnetic orientation ($r=0.652$, modes = 161° and 341°, $P<0.01$, Rayleigh test, $n=10$ birds). *b*, Orientation of the birds (group 2) exposed to the natural clear daytime sky in a magnetic field shifted 90° CCW (magnetic N=geographic W). The group showed E-W magnetic orientation ($r=0.649$, modes = 88° and 268°, $P<0.001$, Rayleigh test, $n=15$ birds). *c*, Orientation of the birds (group 3) exposed to the clear daytime sky in cages covered by pseudodepolarizers in an unshifted magnetic field. This group showed NNW-SSE magnetic orientation ($r=0.773$, modes = 163° and 343°, $P<0.001$, Rayleigh test, $n=12$ birds). *d*, Orientation of the birds (group 4) exposed to the clear daytime sky in cages covered by pseudodepolarizers in a magnetic field shifted 90° CCW. This group showed N-S magnetic orientation ($r=0.651$, modes = 1° and 181°, $P<0.005$, Rayleigh test, $n=12$ birds). Points with a slash mark indicate hopping activity of birds that showed statistically bimodal orientation²⁰. The broken and solid inner circles represent, respectively, the 5% and 1% significance levels for the Rayleigh test. mN, Magnetic north.

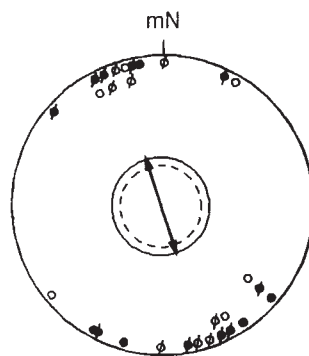
METHODS. Orientation tests were done in a wooden frame garage with an ambient magnetic field of total intensity = 5.4×10^4 nT, inclination = 70°. The shifted field was produced with a 1.8-m square coil^{18,34} and had a total intensity of 5.4×10^4 nT, inclination = 69°. Birds were prevented from seeing outside the test cages by the translucent covers. A dim incandescent light, passing through two diffusers and the translucent cage cover, provided a light of 0.2–0.4 lux in the cages. This variation in light intensity occurred across the array of test cages. Light

intensity in each cage was uniform. Tests began within 1 h after sunset and lasted for 3 h. Roughly half the birds were first tested in the normal field condition, the remainder first tested in the shifted field. Thereafter, individuals were assigned randomly to one or the other test condition. Each point represents the mean of the modal orientation directions of each bird (each bird tested 5–9 times). The orientation records were analysed blind with respect to treatment group and test condition³⁵.

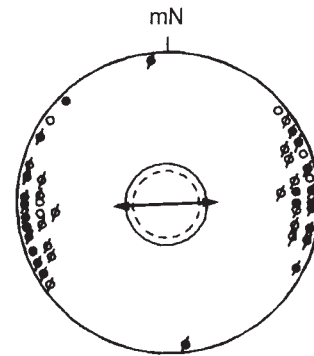
netic N=geographic W); (3) birds that viewed the sky only through a pseudodepolarizer (a sheet of high-order polyester wave-retarding material (Melinix, Polaroid) sandwiched between four sheets of mylar¹⁹) oriented with respect to the Sun's azimuth so as to produce maximal depolarization, within the normal geomagnetic field; and (4) birds exposed only to depolarized skylight within a magnetic field shifted 90° CCW. This experiment was designed to test the hypothesis that polarized light alone provides the calibrating stimulus.

When the birds came into migratory condition for the first time in late September, their directions of magnetic orientation were recorded in covered Emlen funnel orientation cages during tests conducted indoors in normal and shifted magnetic fields (total intensity = 5.4×10^4 nT, inclination = 69°, magnetic N = geographic W). Hand-raised Savannah sparrows typically show axially bimodal migratory orientation when tested over several nights^{5,7,8}. Individual birds usually orient in a unimodal direction in each test, but often switch to more or less opposite directions on subsequent nights. As in previous experiments⁷, Savannah sparrows reared with exposure to the natural daytime sky in an unshifted magnetic field (group 1) developed NNW-SSE magnetic orientation (Fig. 1*a*). Birds that could see the natural sky, including the Sun's disc and skylight polarization patterns only within a shifted magnetic field (group 2), showed dramatically different E-W magnetic orientation (Fig. 1*b*). As in previous experiments^{5,7}, the preferred magnetic direction has been calibrated to correspond to a N-S axis based on geographic compass

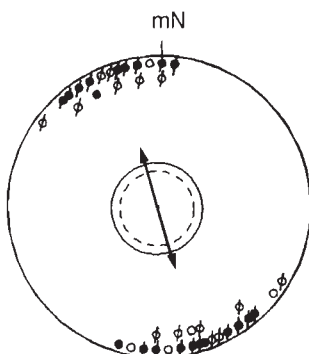
a Natural sky, unshifted field



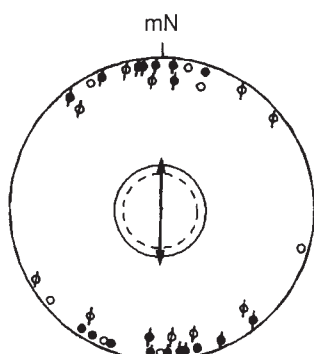
b Natural sky, mN shifted 90° to west



c Depolarized, unshifted field



d Depolarized, mN shifted 90° to west



directions. Birds viewing the clear sky through pseudodepolarizers could see the Sun's disc but were deprived of polarized light produced by Rayleigh scattering as sunlight passes through the atmosphere. Those reared in the normal magnetic field (group 3) produced NNW-SSE magnetic orientation (Fig. 1*c*) indistinguishable from that of the birds that saw the natural sky. Similarly, birds exposed to depolarized skylight in a shifted magnetic field (group 4) showed N-S migratory orientation. The Watson-Williams multisample test²⁰ showed that the mean directions of the four groups differed ($F_{3,45}=24.1$, $P<0.001$). The orientation of the birds exposed to the natural clear daytime sky in the shifted magnetic field (group 2) differed from that of the birds which saw depolarized skylight within the shifted magnetic field (group 4) and from the control groups ($P<0.001$, Watson's U^2 tests)²⁰. The angular difference of 87° between groups 2 and 4 does not differ from the expected and maximum possible difference of 90° (based on 95% confidence interval).

Altered magnetic migratory orientation was observed only in the birds that had visual access to the natural sky, including the Sun itself as well as polarized skylight patterns. The group of birds that viewed the sky through depolarizers within the shifted magnetic field showed no indication of an altered preferred magnetic orientation direction. Birds of all groups had similar opportunity to observe the Sun's position and path across the sky. Although celestial rotation and the location of true north may be assessed by the Sun's path and by both dynamic and static patterns of skylight polarization during daytime^{11,12}, the results

show that polarized skylight is necessary to produce the calibration of the magnetic preference. There is no evidence that the Sun is involved in this process.

Much recent evidence shows that visual information available at sunset is of great importance in the orientation of night-migrating songbirds^{21,22}. Although laboratory experiments designed to test for polarized light sensitivity in the homing pigeon (*Columba livia*) have yielded mixed results²³⁻²⁵, behavioural experiments with migratory birds have consistently indicated that polarized skylight patterns are involved in orientation^{19,26-29}. When directional magnetic information was not available, blackcaps (*Sylvia articapilla*) and European robins (*Erithacus rubecula*) became disoriented when tested at sunset in cages covered with pseudodepolarizers even though the position of the setting sun was clearly visible^{30,31}. Thus polarized skylight patterns, rather than the sun itself, seem to be the daylight stimulus providing information about geographic compass directions that are relevant both in the ontogeny of the orientation system of migratory birds and in day-to-day orientation decision-making during migration. Many vertebrate animals use a time-compensated mechanism based on solar information as a compass³², including pigeons in which it is the primary compass in homing navigation³³. Although it is widely assumed that these 'sun compasses' are based on the Sun's disc, at least in the case of homing pigeons there seems to be no conclusive evidence that the Sun and not polarized skylight provides the relevant stimulus. □

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Red light disrupts magnetic orientation of migratory birds

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THE transduction mechanisms and the neurophysiological basis of magnetoreception in birds are still largely unexplained, even though the role of the magnetic compass in the orientation of birds is fairly well understood¹. The discussion on magnetoreception in birds and terrestrial vertebrates focuses mainly on two mechanisms: small particles of magnetite^{2,3} and biochemical bi-radical reactions of excited macromolecules^{4,5}. When the bi-radical hypothesis was first proposed, magnetic resonance phenomena in the retina were suggested as the primary processes⁴, which led to the question of whether magnetoreception was light-dependent. Homing experiments⁶ and electrophysiological evidence⁷ from pigeons have produced evidence consistent with such a mechanism. An effect of the spectral composition of light on magnetic compass orientation in amphibians has recently been described⁸: under blue light of 450 nm and below, newts oriented as they did under the full spectrum, whereas they showed a roughly 90° counter-clockwise shift when tested under wavelengths at or above 500 nm. Here we report the first orientation tests on migratory birds under light of different wavelengths; the results suggest a light-dependent process that appears to differ from that reported in newts.

The test birds were silvereyes (*Zosterops l. lateralis*), an Australian passerine that shows regular seasonal movements between Tasmania and the Australian mainland. Maximal migration is observed in the twilight hours of dawn and dusk.

The test birds had been mistnetted during the previous winter in Armidale, New South Wales. Laboratory tests in spring revealed that they use the same kind of magnetic inclination compass as described for palaeartic migrants⁹.

Orientation tests under light of different wavelengths were done with the same individuals in the Australian autumn between 10 March and 14 April, 1992. Tests under the full spectrum of a tungsten light bulb ('white') served as control. Details on the coloured lights are given in Table 1, their levels were adjusted to be of equal quantal flux. The light passed through three layers of diffusers before entering the test cages. The birds were tested one at a time, with the assignment of the test condition following a pseudo-random sequence. Their behaviour was recorded in funnel cages lined with typewriter correction paper^{9,10}, and the heading of each recording was calculated, from the distribution of scratches left on the coating of the inclined walls (see Fig. 1).

The data show a clear difference in orientation: under 'white', green and blue lights, the headings lie predominantly in the NNE, whereas no such clustering is found under red light. From the 22 birds tested, 11 contributed between 3 and 6 headings to each test condition, which represent 60% of the control data and between 81% and 86% of the coloured light data. The directions of the individual mean vectors of these birds are indicated in

TABLE 1 Test conditions

Colour	Wavelengths (nm)		Irradiance (W m ⁻²) inside the cage	Light source
	$\lambda_{\max}$	$\lambda/2_{\text{low}}, \lambda/2_{\text{high}}$		
White (control)	From 350 onward, increasing		0.0037	Tungsten light bulb
Red	633	616, 656	0.0027	Diodes, LED
Green	571	555, 588	0.0030	Diodes, LED
Blue	443	402, 472	0.0039	Glass filter, cool beam lamp

Fig. 1 at the outer periphery: the birds significantly oriented in the seasonally appropriate migratory direction except under red light, where this orientation is disrupted. The numerical data are given in Table 2, together with data on activity.

The lower activity observed under blue light was probably due to the fact that the strong light bulb behind the filter slightly raised the temperature in the test cages, which has a damping effect on migratory activity in autumn. The orientation observed under blue light, however, is in no way different from that observed under 'white' or green. The scatter under green light is slightly smaller than under 'white' control conditions. An obvious difference in orientation is found under red light: the vectors of the individual birds are significantly shorter, and the mean headings of the birds are distributed in all directions (see Fig. 1). The distribution of the individual vectors is significantly different from that of control (see Table 2) and also from those obtained under green and blue light ($P < 0.001$).

Thus our data clearly show an effect of the spectral composition of light on the migratory orientation of silveryeyes, but the interpretation of this effect is not easy. Unfortunately, information on the spectral sensitivity of avian eyes is scarce^{11,12}. The only passerine studied so far is the Pekin robin (*Leiothrix lutea*), where peaks of spectral sensitivity under photopic conditions were found at 350 nm, 460 nm, 530 nm and 620 nm¹³. It is unclear to what extent these findings can be generalized. The possibility that our test birds could not perceive the long wavelength seems improbable in view of the closeness of the 620 nm peak to the 633 nm used in our tests, and in view of the birds' activity under red light, as total darkness usually leads to a suppression of migratory activity¹⁴. The level and concentration of activity observed under 'red' which is not different from control (see Table 2), suggests normal migratory behaviour and speaks against reduced motivation. Thus red light around 633 nm seems to be visible, allowing normal activity, but without allowing meaningful orientation.

So far, two hypotheses exist for the interpretation of light-dependent responses to the magnetic field. Leask's hypothesis⁴ is based on an optical pumping mechanism: by photon absorption, molecules enter a singlet excited state, from which they may reach a triplet state through excitation transfer. The triplet state, in contrast to the singlet states, has a magnetic moment and, under suitable conditions, will elicit processes leading to magnetic resonance. The excitation transfer probability depends on the energy of the photons. Leask⁴ proposed that monochromatic light at long wavelengths might modify it in a way which interferes with magnetoreception. Phillips and Borland⁸, by contrast, suggested that certain wavelengths excite magnetoreceptors with different spectral sensitivity which produce antagonistic input in second-order cells. This may lead to a 90° rotation of the pattern

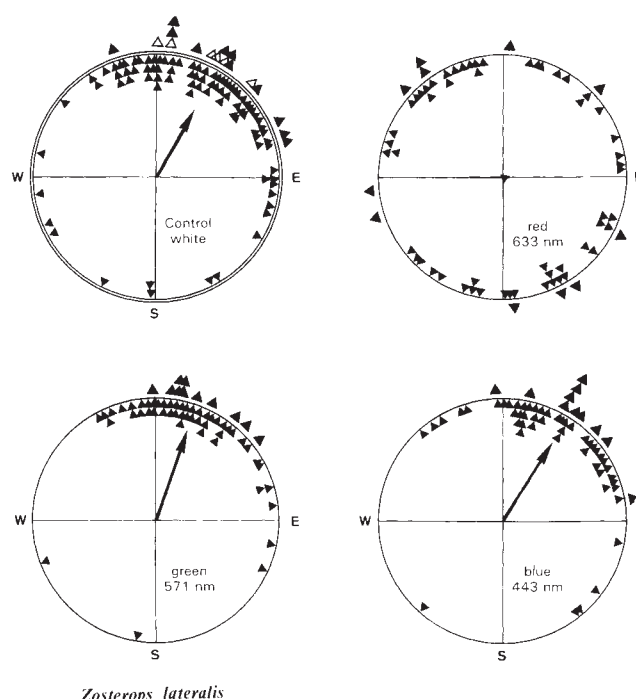


FIG. 1 Orientation behaviour of migratory silveryeyes tested in light of various spectral compositions. Headings of single recordings are given as triangles inside the circle, the arrows represent the mean vectors drawn proportional to the radius of the circle. The solid symbols outside the circle indicate the mean headings of the 11 birds that contributed between 3 and 6 data points to each test condition. The mean headings of birds contributing 3 to 6 data points to the control, but fewer to the other samples are given as open symbols in the upper left diagram.

if the input is inverted by presenting light of long wavelengths only. The latter authors refer to their recent findings on the red-spotted newt (*Notophthalmus viridescens*) where wavelength from 500 nm to 600 nm produced such a deflection, whereas short wavelength of 450 nm and below led to results that were indistinguishable from the response under the full spectrum. At intermediate wavelengths of 475 nm, they found random behaviour⁸.

The responses of our test birds under a similar wavelength range, however, show a different pattern. Orientation under green and under blue light did not differ in any respect; that is a directional shift was not observed, although the 571 nm green light and the 443 nm blue light may have stimulated different receptors or combinations of receptors. The remote possibility that different reactions occur at other wavelengths not yet tested cannot be excluded completely. But our present data, in particular the lack of orientation under 633 nm, rather suggest an 'all or none' process: orientation seems to require photons of a certain minimum threshold of energy, but if that level is exceeded, the actual wavelength does not appear to influence the response any further.

In view of the role of light in magnetoreception, there are at least three different mechanisms indicated in vertebrates: the two different light-dependent processes found in amphibians and birds, and the light-independent processes of turtles¹⁵, rodents¹⁶ and possibly salmon¹⁷. The latter need not necessarily be identical, as salmon¹⁸ and rodents¹⁶ seem to have a polarity compass, whereas turtles have an inclination compass¹⁹. If this diversity of mechanisms proves true, it might suggest independent development of magnetic receptors in various groups of vertebrates. At the moment, however, very few species have been studied in behavioural experiments, and even fewer in electrophysiological experiments. More information on the nature of magnetic

TABLE 2 Activity and orientation of the 11 active birds

Colour	Level	Activity		Median r_{ind}	Second order mean		Difference from control
		Concentration			α_N	r_N	
Control	390	0.42		0.74	34°	0.63***	
Red	203 (NS)	0.29 (NS)		0.39**	182°	0.08 (NS)	**
Green	352 (NS)	0.39 (NS)		0.94*	21°	0.84***	*
Blue	180**	0.38 (NS)		0.89 (NS)	32°	0.76***	(NS)

Activity level is the median value of median number of scratches per bird; activity concentration is the median value of median concentration of activity within the test cage per bird; median r_{ind} is the median value of length of individual vectors of birds, calculated by vector addition from each bird's headings. Asterisks at these data indicate significant difference from the control data by the Mann-Whitney U-test. α_N , r_N is the direction and length of second-order mean vector based on the individual mean vectors of the 11 birds; asterisks at r_N indicate a significant common directional tendency by the Hotelling test²⁰. The last column indicates whether the distribution of the individual mean vectors differs from that observed under control by Mardia's two sample test for bivariate samples²⁰. Significance levels: NS, not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

responses, their neurobiological basis and the role of light in other vertebrate species is urgently needed. □

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Auditory illusions and the single hair cell

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LIKE our other senses, the auditory system can produce illusions. Prominent among these are distortion products^{1–5}: when listening to two tones, one of frequency f_1 and the second of a higher frequency f_2 , an individual may hear not only these primary tones, but also a difference tone of frequency $f_2 - f_1$, a sum tone of frequency $f_2 + f_1$, and combination tones of frequencies such as $2f_1 - f_2$ and $2f_2 - f_1$. Discovered by Tartini early in the eighteenth century^{6,7}, these illusory sounds are sufficiently conspicuous that they were employed to carry melodies in classical compositions. Distortion products originate within the cochlea, where they manifest themselves in the basilar membrane's vibration⁸. Here we demonstrate distortion products in individual hair cells of the bullfrog's sacculus, where they emerge from a nonlinearity inherent in the mechano-electrical transduction process. In addition to offering an explanation for cochlear distortion products, our results suggest that the mechanical properties of hair bundles significantly influence the basilar membrane's motion.

Sound stimulates a hair cell by deflecting its mechanically sensitive hair bundle, which transduces this stimulus into an electrical response^{9–11}. Previous work on hair cells of the bullfrog's sacculus indicates that deflection of a hair bundle acts through elastic gating springs to open mechanically sensitive ion channels^{10–12}. As a result, a portion of the force (F) necessary to displace a hair bundle through a given distance (X) reflects the work done in pulling a channel's gates ajar. If a channel has three states^{12–14}, two closed and one open, then

$$F = (\kappa_S + \kappa_G)X + C + [p_1(\Delta\kappa X + Nz_{12}) - p_3Nz_{23}] \quad (1)$$

in which κ_S and κ_G are the stiffnesses of the tilting stereocilia and the combined gating springs respectively, N is the number of channels in a hair bundle, and C embraces several constant terms^{10,15}. The probability that a channel is in the first closed state or in the third, open state is respectively p_1 or p_3 , and $\Delta\kappa$ is the difference in stiffness between these two states. A channel's sensitivities to bundle deflection, z_{12} and z_{23} , express the change in force when a channel switches from the first to the second closed state, or from the second closed state to the open state. Both of the probabilities for transitions between states are monotonic functions of hair-bundle displacement.

As indicated by equation (1), bundle deflection and the ensuing force are linearly related when the bundle is displaced extensively in the positive direction or negative direction; the bundle's stiffness is nearly constant under these conditions. Over the narrow range of bundle motions in which channels switch from

state to state, though, the last term asserts itself: for displacements within about ± 40 nm of the resting position, the bundle's stiffness is smaller and varies with displacement. This localized softening of the hair bundle, called gating compliance, has been demonstrated both in anuran¹⁵ and in mammalian hair cells¹⁶.

The final term in equation (1) renders the relation between hair-bundle displacement and force nonlinear. Hypothesizing that this nonlinearity underlies distortion products, we sought to identify these products at the single-cell level by stimulating the bundles of individual hair cells from the bullfrog's sacculus^{15–17}. When moved back-and-forth at a single frequency, a hair bundle exerted force against a stimulus fibre at only that frequency and its second harmonic (Fig. 1a). When a second frequency was added to the displacement command, however, a more complex response arose (Figs 1b and 2b). In addition to forces at the two primary frequencies and their second harmonics, the bundle produced forces at the difference and sum frequencies ($f_2 - f_1$ and $f_1 + f_2$) and at combination frequencies ($2f_1 - f_2$ and $2f_2 - f_1$). These distortion products occurred in all 30 responsive hair cells investigated, for a variety of primary frequencies, and for various ratios of f_2 to f_1 . We investigated the distortion products over a broad range of stimulus amplitudes up to ± 125 nm. Some of these products, for example the cubic difference product ($2f_1 - f_2$), occurred with stimuli as small as ± 12 nm. The distortion products accordingly were not dependent on saturating levels of stimulation, but arose from a nonlinearity whose effects were felt at near-threshold levels of stimulation¹. In their distribution and relative magnitudes, the distortion products resembled those observed upon the basilar membrane⁸.

To rule out the possibility that the distortion products observed *in vitro* originated from nonlinearities in the apparatus, we performed four control experiments. Distortion products were detected neither upon deflection of inert objects such as glass fibres, nor during stimulation of hair bundles on damaged cells that lacked gating compliance. When a bundle's transduction channels were irreversibly inactivated by disruption of the gating springs^{18,19}, all the distortion products promptly vanished (Fig. 1c). Iontophoretic application of gentamicin²⁰, a transduction-channel blocker²¹, had a similar but reversible effect on distortion products.

If gating compliance underlies distortion products, it should be possible to predict both the frequencies and the amplitudes of the products from the measured mechanical properties of a hair bundle. After measuring the relation between a bundle's displacement and the consequent restoring force¹⁵ (Fig. 2a), we elicited distortion products by subjecting the same hair bundle to sinusoidal stimulation at two frequencies (Fig. 2b). We then used equation (1) to calculate the force expected at any instant during stimulation by concurrent displacements at the two frequencies. The theoretical power spectrum of force components (Fig. 2c) accorded well with the experimental observations (Fig. 2b). A similar quantitative agreement was obtained for each of the three additional hair cells studied in detail, while the results

from all 30 cells were qualitatively consistent. The agreement between the theoretical predictions and experimental results is especially compelling in light of the fact that the former were calculated with no free parameters.

We have demonstrated mechanical distortion products *in vitro* and quantitatively accounted for their origin over the range of hair-bundle displacements associated with gating compliance in hair cells of the bullfrog's sacculus¹⁵. If gating compliance is to explain distortion products in the cochlea, sound of the intensities that elicit these products must produce hair-bundle motions of comparable size. Distortion products of cochlear origin are readily audible over a roughly 180-fold range of stimulus amplitudes^{3,22}, at sound-pressure levels from 20 dB to 65 dB. Based upon the basilar membrane's sensitivity to sound²³ and

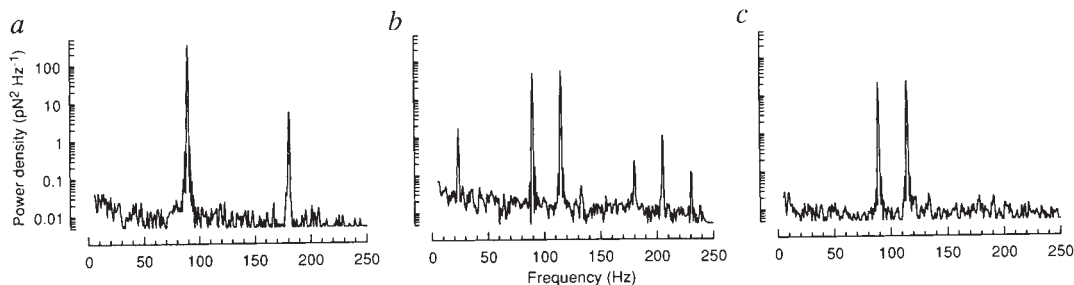
the geometrical relation between basilar-membrane vibration and hair-bundle motion²⁴, a 20-dB stimulus displaces hair bundles by about ± 2 nm, and a 65-dB sound moves bundles by no more than ± 25 nm. When two tones are presented together at a frequency ratio typical of psychophysical experiments^{1,2,4}, hair bundles that respond to both stimuli undergo displacements considerably smaller than the sum of the maximal motions of hair bundles at the characteristic places. We conclude that the stimuli employed to evoke distortion products deflect cochlear hair bundles by no more than a few tens of nanometres, and hence that the bundle motions lie in the range of the gating compliance.

The origin of distortion products from gating compliance has an important implication for studies of auditory frequency tun-

FIG. 1 Distortion products

in the force produced by a hair bundle. **a**, When moved back and forth at a frequency of 90 Hz, a hair bundle produced measurable force only at the stimulus frequency and second-harmonic frequency, 180 Hz. **b**, When stimulated with two displacement components of identical amplitude and at frequencies of 90 Hz and 115 Hz, the same bundle exerted forces, not only at the two primary frequencies, but also at the frequencies of several distortion products: 25 Hz ($f_2 - f_1$), 180 Hz ($2f_1$), 205 Hz ($f_1 + f_2$) and 230 Hz ($2f_2$). **c**, Immediately after its transduction apparatus was destroyed, the bundle displayed only the force components corresponding to the primary stimulus frequencies.

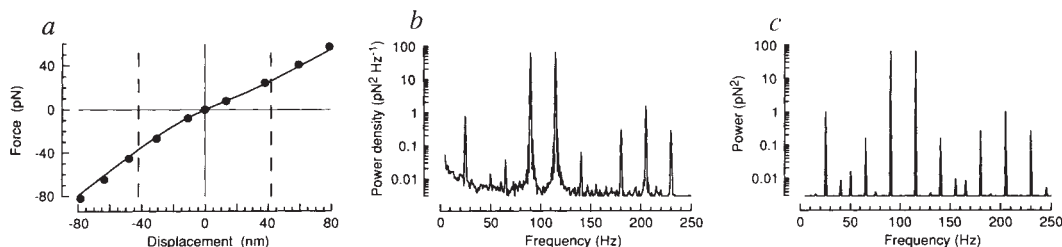
METHODS. The saccular macula of the bullfrog's internal ear was dissected and maintained at room temperature in saline solution³⁰ containing either 100 μM (Fig. 1) or 4 mM Ca^{2+} (Fig. 2). An individual hair bundle was attached near its tip to the tip of a horizontal, metal-coated glass fibre about 100 μm in length and 0.8 μm in diameter, whose motion was measured with a calibrated, dual-photodiode displacement monitor¹⁵. A displacement-clamp system¹⁹ ensured that the fibre's tip underwent the commanded pattern of displacement, either one sinusoidal motion of ± 34 nm or the sum of two sinusoidal motions of differ-



ing frequencies, each of ± 14 nm. The clamp system measured the force required to effect the commanded motion, which was equal and opposite to the force exerted by the hair bundle. Because the gating of transduction channels in hair cells is not voltage-sensitive³⁶, we allowed the cell's membrane potential to vary during stimulation. After force records were subjected to Fourier analysis, the results were plotted as one-sided power spectra. The primary frequencies were selected on two grounds: adaptation by saccular hair cells diminishes their responsiveness³⁷ below ~ 20 Hz, and the frequency response of the displacement clamp¹⁹ leads to progressively greater underestimation of force at frequencies above 160 Hz. After the force records for **a** and **b** were obtained, but before the data in **c** were acquired, we obliterated the hair bundle's tip links³⁸, the morphological correlates of the gating springs, by iontophoretic application of a Ca^{2+} chelator¹⁸.

FIG. 2 Comparison of

experimental and theoretical distortion products. **a**, The relation between bundle displacement and force was determined by deflecting a bundle with stimulus pulses and measuring the flexion of the stimulus fibre¹⁵. The data points of this nonlinear relation were fitted to an analytical expression (equation (1); V.S.M. and A.J.H., manuscript in preparation) based on a three-state model¹²⁻¹⁴. The bundle's maximal excursion during the combined stimulation employed for the experiment in **b** is indicated by dashed lines. **b**, Concurrent stimulation of the same hair bundle with ± 21 -nm sinusoidal stimuli at 90 Hz and 115 Hz elicited a wealth of distortion products: 25 Hz ($f_2 - f_1$), 50 Hz ($2f_2 - 2f_1$), 65 Hz ($2f_1 - f_2$), 140 Hz ($2f_2 - f_1$), 180 Hz ($2f_1$), 205 Hz ($f_1 + f_2$) and 230 Hz ($2f_2$). **c**, Distortion products of similar frequency and magnitude arose in the power spectrum calculated from the bundle's measured stiffness and gating compliance. The primary frequencies and stimulus amplitudes used in the simulation were the same as those employed in **b**, and the scaling of the figures is identical to facilitate their comparison.



METHODS. From the fit in **a**, we inferred that this hair bundle contained $N=130$ active transduction channels, whose mechanical sensitivities were $z_{12}=200$ fN and $z_{23}=100$ fN; the corresponding transition mid-points were $X_{12}=-20$ nm and $X_{23}=50$ nm. The total stiffness of the bundle's stereociliary pivots was $\kappa_s=550$ $\mu\text{N m}^{-1}$, that of the gating springs was $\kappa_g=300$ $\mu\text{N m}^{-1}$, and $\Delta\kappa=450$ $\mu\text{N m}^{-1}$. The single-sided power spectrum in **b** was obtained experimentally from a force record by the methods described in Fig. 1 legend. Substituting into equation (1) a displacement consisting of two sinusoidal stimuli, each of ± 21 nm, we derived an explicit expression for the expected force as a function of time. To predict the power at each of the primary and distortion-product frequencies, we evaluated equation (1) and performed Fourier analysis of this oscillatory force.

ing. When a complex sound is analysed by the cochlea, each frequency component evokes a travelling wave whose amplitude peaks at a frequency-dependent position along the basilar membrane. The site of this peak is determined in large part by the stiffness of the basilar membrane²⁵⁻²⁷, which is assumed to be dominated by the elasticity of its connective tissue layers²⁶. If hair bundles account for distortion tones, however, they too must exert an influence on the basilar membrane's mechanical impedance. The conclusion that hair bundles contribute significantly to the impedance of receptor organs, which is supported by direct measurements in the bullfrog's sacculus²⁸, is not surprising. For maximal efficiency, the ear should direct as much as possible of the energy in a sound to the mechanically receptive hair bundles. It follows that the bundles should constitute an appreciable part of the mechanical impedance to basilar-membrane motion, and that distortion-product forces produced by hair bundles should reciprocally produce propagating distortion products²⁹ in basilar-membrane motion. By the same logic, hair bundles may actively enhance the basilar membrane's motion, for they contain myosin (ref. 30, and P. G. Gillespie, M. C. Wagner and A.J.H., manuscript in preparation) and are capable of producing both spontaneous and evoked movements^{15,17,31,32}. Hair bundles may by this means contribute to the amplificatory process that underlies the cochlea's great sensitivity¹¹ and is thought to originate principally from the contractile activity of outer hair cells^{33,34}.

It seems baffling at first blush that our hearing should be perturbed by a nonlinearity substantial enough to evoke audible distortion products. If these products arise from gating compliance, however, their presence is less obscure: they are an inevitable price paid for a sensory system in which mechanical input is directly coupled to the gating of transduction channels. Natural selection has evidently found the price acceptable, presumably because this mechanism of transduction is both remarkably fast³⁵ and extraordinarily sensitive³². □

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Galanin regulates basal and oestrogen-stimulated lactotroph function

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OESTROGEN, an essential physiological regulator of reproductive function¹, controls lactotroph proliferation and prolactin release². The neuropeptide galanin co-localizes to the lactotroph³, but its physiological function is largely unknown. Pituitary galanin expression is extremely sensitive to the oestrogen status of the animal. A marked elevation occurs during pregnancy and lactation⁴, and exogenous 17 β -oestradiol can cause a 4,000-fold increase in messenger RNA levels⁵. Here we report that galanin is secreted by a minority of lactotrophs and is essential for the regulation of basal and vasoactive-intestinal-polypeptide-stimulated prolactin release. Hyperoestrogenization increases the number of galanin-secreting cells and the resulting increase in basal prolactin release is completely abolished by treatment with galanin antiserum. Galanin is a potent lactotroph growth factor and galanin-immunoneutralization completely inhibits the previously reported⁶⁻⁸ mitogenic effects of oestrogen on the lactotroph. These findings represent direct evidence for paracrine regulation of lactotroph function and demonstrate that the effect of oestrogen on lactotroph proliferation and prolactin release are mediated by locally secreted galanin.

The neuropeptides galanin and vasoactive intestinal polypeptide (VIP) are synthesized and stored in the anterior pituitary^{5,9,10} and stimulate the release of prolactin in a number of *in vivo* and *in vitro* systems¹¹⁻¹³. Galanin is predominantly localized to the lactotroph in female rats³, but localization of VIP to the lactotroph in the rat³ has not been confirmed^{14,15}. The role galanin and VIP play in lactotroph function was studied using fluorescence-activated cell sorted (FACS) enriched primary lactotrophs and the 235-1 rat clonal lactotroph cell line¹⁶. Immunoneutralization using antisera raised against galanin¹⁷ (G_{as}) and VIP¹⁸ (V_{as}) was performed on dispersed anterior pituitary cells. Both G_{as} and V_{as} inhibit basal prolactin secretion (88 $\pm$ 5.1% and 55 $\pm$ 2.7%, respectively; Fig. 1a). Exogenous galanin and VIP stimulate prolactin secretion to the same extent. G_{as} abolishes VIP-stimulated prolactin release, but V_{as} does not modulate galanin-stimulated prolactin release. Parallel experiments using 235-1 cells demonstrate responsiveness to galanin and G_{as} but not to thyroliberin, VIP or V_{as} (Fig. 1b).

Using the galanin cell blot assay, 9 $\pm$ 1% of all lactotrophs are galanin secretors. Galanin secretors are not identified in the lactotroph-depleted population. All 235-1 cells secrete galanin at a low level in the cell blot assay and this was quantified by specific radioimmunoassay¹⁹ (10.3 $\pm$ 0.85 fmol per 10⁶ cells).

Galanin increases thymidine incorporation into confluent, quiescent 235-1 cells by 315 $\pm$ 25% (P < 0.001) and stimulates cell proliferation in log growth phase by 196 $\pm$ 9% (P < 0.001). Basal proliferation rate is reduced by 51 $\pm$ 6% (P < 0.001) after exposure to galanin antisera (Fig. 2).

Prolactin secretion from FACS-enriched lactotrophs was compared to unseparated dispersed pituitary cells. Inhibition of basal prolactin release by V_{as} is abolished, and the enriched lactotrophs are supersensitive to the addition of VIP (Fig. 3), consistent with VIP release by a cell type other than the lactotroph. Using FACS, lactotrophs were separated into galanin-secreting and non-galanin-secreting subpopulations (see methods, for Fig.

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3) and prolactin release measured by RHPA. The galanin secretors retain a normal response to thyroliberin but are unresponsive to the addition of galanin, VIP, G_{as} or V_{as} (Fig. 3). VIP stimulates galanin secretion, causing an increase in galanin blot area by 2.15 ± 0.23 -fold compared with unstimulated galanin secretors ($P < 0.01$). The increase in galanin release may act on the non-galanin secreting cells to stimulate prolactin secretion, thus explaining the prolonged delay observed in the action of VIP²⁰⁻²².

Basal prolactin release from the non-galanin secreting lactotrophs is significantly lower than from the galanin secretors (mean plaque area, $0.028 \pm 0.003 \mu m^2$ versus $0.049 \pm 0.006 \mu m^2$;

$P < 0.01$), emphasizing the dependence of these cells on galanin. The non-galanin-secreting lactotrophs are supersensitive to the addition of galanin and unresponsive to VIP, G_{as} and V_{as} (Fig. 3).

The effects of oestrogen status on galanin regulation of lactotroph function were studied using endocrine-manipulated animals. Ovariectomy reduces the number of galanin-secreting cells to below the detectable limit of the blot assay, whereas hyperoestrogenization increases the number of secretors to $39 \pm 2\%$ of all lactotrophs. Mean galanin blot area is unchanged in the hyperoestrogenized state compared with control animals ($0.011 \pm 0.002 \mu m^2$ versus $0.013 \pm 0.003 \mu m^2$; $P > 0.05$). The

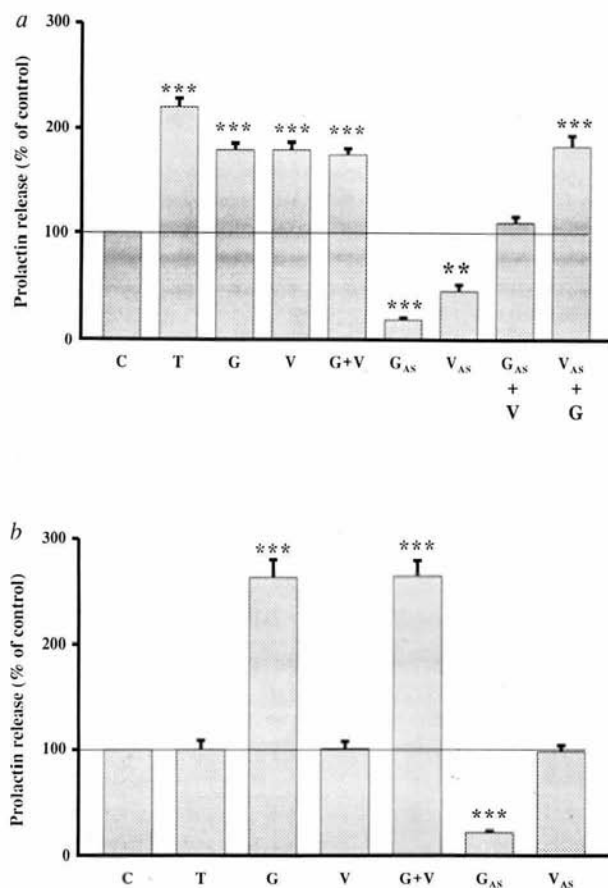


FIG. 1 Prolactin secretion from a, unenriched dispersed anterior pituitary cells measured by reverse haemolytic plaque assay, and b, 235-1 clonal lactotroph cell lines, expressed as a percentage of basal release in response to thyroliberin (T), galanin (G), vasoactive intestinal polypeptide (V), or galanin antisera, used at a final dilution of 1/100 (G_{as}), or vasoactive intestinal polypeptide antisera, used at a final dilution of 1/100 (V_{as}). In a, antisera were also used in combination with galanin or vasoactive intestinal polypeptide. All peptides were used at 100 nM. Results are expressed as a mean $\pm$ s.e.m. of 5 separate experiments. Statistical analysis was done using ANOVA. *** $P < 0.001$; ** $P < 0.01$; and * $P < 0.05$.

METHODS. Non-pregnant randomly cycling female Wistar rats were used for all experiments. As previously described²⁵, anterior pituitaries were dispersed to a single cell population. Cells were cultured for 48 h and prolactin secretion from single cells then assessed using a reverse haemolytic plaque assay²⁶. The polyclonal antisera raised in rabbits against rat galanin and rat vasoactive intestinal polypeptide used in immunoneutralization experiments have been validated at this dilution²⁴. 235-1 cells were provided by J. C. Porter. Cells were dispersed for subculture by incubation with versene solution containing 0.005% trypsin. Cells were washed twice in serum-free medium before incubation for 2 h in test materials. Medium was then withdrawn and prolactin secretion assayed by ELISA²⁶.

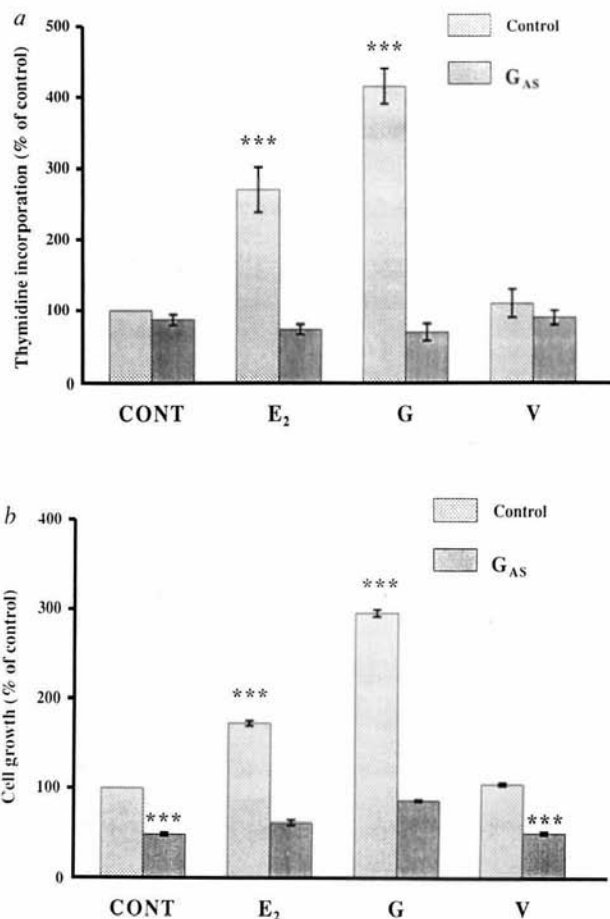


FIG. 2 a, Thymidine incorporation into 235-1 cells, and b, 235-1 cell proliferation was measured. Results are expressed as a percentage of basal incorporation in response to 17β -oestradiol (E_2 , 50 μM), galanin (G), or vasoactive intestinal polypeptide (V). Experiments were repeated in the presence of galanin antisera, used at a final dilution of 1/100 (G_{as}). Peptides were used at a concentration of 100 nM. Results are expressed as a mean $\pm$ s.e.m. of 5 separate experiments. Statistical analysis was performed using ANOVA. *** $P < 0.001$.

METHODS. a, Confluent cells were maintained for 24 h in serum-free medium, and then incubated with [3H]thymidine and test substance for 24 h. Cells were washed twice and DNA extracted following 3 washes with 10% trichloroacetic acid and incubation with 2M perchloroacetic acid at 60 °C for 45 min. Incorporation of [3H]thymidine into DNA was measured by scintillation counting. The amount of protein per well was determined and thymidine incorporation expressed per mg protein. To obviate the possibility that the observed increase in thymidine incorporation was due to any mitogenic effect of prolactin²⁷, these experiments were repeated in the presence of prolactin antiserum (data not shown), which had no effect on basal or stimulated thymidine incorporation. b, After 48 h in culture, cells were incubated with test substances in serum free medium for 24 h. Culture medium was then removed and cell number measured using the MTT dye colorimetric method²⁸.

absence of endogenous galanin secretion in the ovariectomized animal would explain the observed decrease in basal prolactin secretion to $28 \pm 3.2\%$ of control ($P < 0.01$). The lack of locally secreted galanin in the ovariectomized lactotrophs might account for their decreased responsiveness to the addition of thyroliberin, VIP, G_{as} and V_{as} (Fig. 4a). The response to exogenous galanin is increased in these cells to a degree similar to that observed in the non-galanin-secreting lactotrophs from the control group of animals (Fig. 3). Hyperoestrogenization significantly increases basal prolactin release (2.4 ± 0.18 fold; $P < 0.01$). Oestrogen treatment also decreases the response to the addition of thyroliberin, galanin and VIP, which may be explained by high endogenous galanin secretion. Hyperoestrogenized lactotrophs are unresponsive to the addition of V_{as} but very sensitive to G_{as} (Fig. 4a), which markedly decreases prolactin release compared to control animals ($P < 0.001$). 17β -oestradiol increases thymidine incorporation into 235-1 cells by $170 \pm 38\%$ ($P < 0.001$) and cell proliferation by $72 \pm 3\%$ ($P < 0.001$). These effects are abolished by co-incubation with galanin antisera (Fig. 2). Thus in states of high oestrogen expo-

sure, lactotroph proliferation and basal prolactin release are galanin-dependent events.

In summary, our observations show that galanin is a regulator of lactotroph function (Fig. 4b) and is an important mediator of oestrogen-induced growth in the rat pituitary, supporting the concept of paracrine regulatory mechanisms and offering an explanation for the highly conserved topographical arrangement of the pituitary gland²³.

Exposure to 17β -oestradiol has been reported to cause up to a 1,000-fold rise in galanin expression in rat prolactinomas and implantable lactotroph clonal cell lines^{5,10}. Chronic hyperoestro-

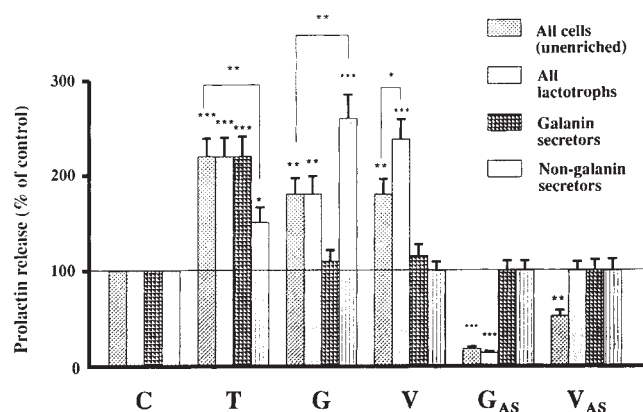


FIG. 3 Prolactin secretion from unenriched dispersed cells was compared to the entire population of FACS-enriched lactotrophs, galanin- and non-galanin-secreting lactotrophs. Results were expressed as a percentage of basal release in response to thyroliberin (T), galanin (G), vasoactive intestinal polypeptide (V), galanin antisera, used at a final dilution of 1/100 (G_{as}), or vasoactive intestinal polypeptide antisera, used at a final dilution of 1/100 (V_{as}). All peptides were used at 100 nM. Results are expressed as a mean $\pm$ s.e.m. of 5 separate experiments. Statistical analysis was performed using ANOVA. *** $P < 0.001$; ** $P < 0.01$; and * $P < 0.05$.

METHODS. Galanin secretion from single lactotrophs was measured using an adaptation of the standard cell blot assay²⁹. Chambers were constructed using microscope slides, 2×2 cm squares of polyvinylidene difluoride transfer membrane (Imobilon PVDF, Millipore), double-sided sticking tape and coverslips. Dispersed cells (10^4 in 100 μ l DMEM; Gibco) were added to the chambers and then incubated for 6 h at 37 °C. Transfer membranes were then incubated with 0.15% phenylhydrazine hydrochloride (Sigma) followed by 1% BSA in PBS. After the blocking procedure, galanin bound to the membrane was immunostained with (1) an antiserum raised in rabbits against rat galanin⁹, used at a final dilution of 1:1,000, for 18 h at 4 °C; (2) goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma), at a final dilution 1:1,000, for 1 h at room temperature; (3) rabbit peroxidase-antiperoxidase (Sigma), 1:2,000, for 1 h at room temperature; (4) 3,3'-diaminobenzidine tetrahydrochloride (Sigma), 0.05% in 0.01M citrate buffer, pH 5.2, as chromogen, initiated with 0.03% hydrogen peroxide for 30 min at room temperature. Lactotrophs were stratified by FACS on the basis of the intensity of cell surface prolactin immunofluorescence and then sorted into fractions of 10%. The 10% of lactotrophs expressing the least cell-surface prolactin were 89 $\pm$ 5% pure galanin secretors in the cell blot assay. The remaining 90% of lactotrophs, expressing a greater amount of cell-surface prolactin, did not secrete galanin in the blot assay.

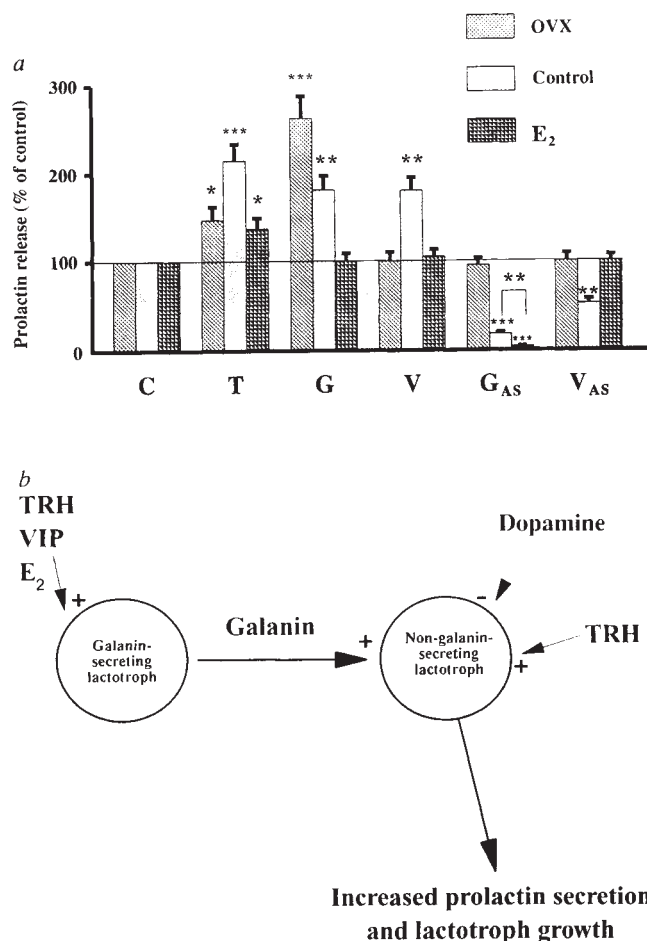


FIG. 4 a, Prolactin secretion from control, hyperoestrogenized (E_2) and ovariectomized (OVX) dispersed anterior pituitary cells was measured by reverse haemolytic plaque assay and expressed as a percentage of basal release in response to thyroliberin (T), galanin (G), vasoactive intestinal polypeptide (V), galanin antisera, used at a final dilution of 1/100 (G_{as}), or vasoactive intestinal polypeptide antisera, used at a final dilution of 1/100 (V_{as}). All peptides were used at 100 nM. Results are expressed as a mean $\pm$ s.e.m. of 5 separate experiments. Statistical analysis was performed using ANOVA. *** $P < 0.001$; ** $P < 0.01$; and * $P < 0.05$. b, Schematic representation of the possible relationships of galanin- and non-galanin-secreting lactotrophs based on data presented here. Vasoactive intestinal polypeptide (VIP), thyroliberin (TRH), and oestrogen (E_2) act principally to increase the release of galanin, thus increasing prolactin release and stimulating lactotroph growth. The effect of dopamine on galanin release has not been studied but is included for completeness.

METHODS. Female Wistar rats were either surgically ovariectomized three weeks before study or hyperoestrogenized by injection with 0.2 mg 17β -oestradiol (dissolved in linseed oil) daily for three weeks. Controls were either sham-operated or injected with vehicle daily for the same period of time as the hyperoestrogenized animals. Animals were then killed, the pituitaries dispersed into single cells and cultured for 48 h before study in the reverse haemolytic plaque assay.

genization in rats first induces lactotroph hyperplasia, followed by adenoma formation and finally the development of prolactinomas^{5,6,8,10}. We have recently characterized a novel galanin receptor in the anterior pituitary and demonstrated that existing galanin antagonists do not bind to it and thus do not affect prolactin secretion²⁴. As the hyperoestrogenized lactotroph seem to be dependent on galanin for both growth and prolactin secretion, a specific pituitary galanin antagonist might be of benefit for the treatment of prolactinomas. □

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Myogenin gene disruption results in perinatal lethality because of severe muscle defect

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MYOGENIN is a member of the basic helix–loop–helix (bHLH) gene family and converts multipotential mesodermal cells to myoblasts^{1–4}. The four members of the *myoD* family show unique spatio-temporal expression patterns⁵ and therefore may have different functions during myogenesis. Here we inactivate the myogenin gene in order to understand its role in myogenesis. Homozygous mutations are lethal perinatally owing to the resulting major defects in skeletal muscle. The extent of disorganization of muscle tissue differs in three regions. In the latero–ventral body wall, most cells, including myogenic cells, disappear and there is rapid accretion of fluid. In the limbs, cells of the myogenic lineage exist, but they are severely disrupted, and some of them are mononucleate with properties of myoblasts. In contrast, there are many axial, intercostal and back muscle fibres to be seen, although fibres are mainly disorganized and Z-lines are not present in most myofibrils. These findings are evidence that myogenin is crucial for muscle development *in utero* and demonstrate that other members of the myogenic gene family cannot compensate for the defect.

Homologous recombination-mediated gene targeting⁶ was achieved with a myogenin gene replacement vector (Fig. 1a). This vector has the *neo* gene inserted into the *SacI* restriction site located in exon 1, upstream of the bHLH domain⁷ to inactivate the myogenin protein, because the reading frame is disrupted in exon 1. From fifty doubly-resistant embryonic stem (ES) cell clones, five homologous recombinants were identified and used for injections into blastocysts. The mutated allele will be referred to as *MyoG^{mt-1}*. Chimaeric male mice generated from injections with one cell line transmitted the ES genome through the germ line after breeding with BALB/c females. Among the

offspring of 40 mice, 12 were wild-type, 28 were heterozygous for the *MyoG^{mt-1}* allele, but none was homozygous for the mutated allele. The predicted number of homozygous mutant individuals was, however, found in embryos dissected during embryonic and perinatal stages. The genotypes of these embryos were as follows: 20 were wild-type, 54 were *MyoG^{mt-1}/+*, and 24 were *MyoG^{mt-1}/MyoG^{mt-1}*. From these results, we concluded that mice carrying the homozygous mutation are perinatal lethal and mice heterozygous for the *MyoG^{mt-1}* allele are indistinguishable from their wild-type littermates and have no apparent phenotype. Distinct myogenin messenger RNA was detected on northern blots from embryos that were wild-type or heterozygous, but not from homozygous mutant embryos. In addition, an aberrant short RNA, probably driven from the promoter region of the *neo* cassette, was faintly observed in heterozygous and homozygous mutant embryos (Fig. 1h). It is unlikely, however, that a functional myogenin protein can be generated from this transcript because (1) myogenin-encoding sequences are downstream of the multiple translation stop codons, (2) an in-frame ATG codon available for translational initiation to synthesize a truncated or chimaeric myogenin is not seen in the corresponding sequences, and (3) mutant myogenic cells are not stained with an anti-myogenin antibody² (Fig. 4B, b).

It has been reported that (1) myogenin is preferentially expressed during the differentiation from myoblasts to myotubes in culture², and (2) myogenin is the predominant factor when fusion from myoblasts to myotubes begins *in utero*⁸. So to characterize the importance of myogenin in the terminal differentiation process, we first examined the embryos at about 13 days post coitum (p.c.), the stage at which myotube formation occurs⁹. Based on external morphology, size, colour and shape, mutant embryos of 14.5 days p.c. and at earlier stages are indistinguishable from those of wild-type littermates. Significant defects are found, however, in skeletal muscles in tissue sections. Although a considerable number of intercostal and axial muscle fibres are observed at 13.5 and 14.5 days p.c. (Fig. 2C, b and c), these fibres are a mixture of fibres at different stages of maturation. Some fibres arranged in parallel arrays appear to be indistinguishable from those of wild-type littermates (Fig. 2C, a), whereas others appear shorter and disorganized. Electron microscopy demonstrates the abnormality of mutant muscle fibres. Z-lines are rarely evident in myofibrils, and even when they can be seen, they are irregular and faintly stained (Fig. 3). In addition to axial and intercostal muscles, abnormal back muscles can be observed in the dorsal region of the neck and upper thorax (Fig. 2C, b). Most of these muscle fibres have degenerated or become disorganized during development. On the other hand, in latero–ventral regions of body wall, most cells

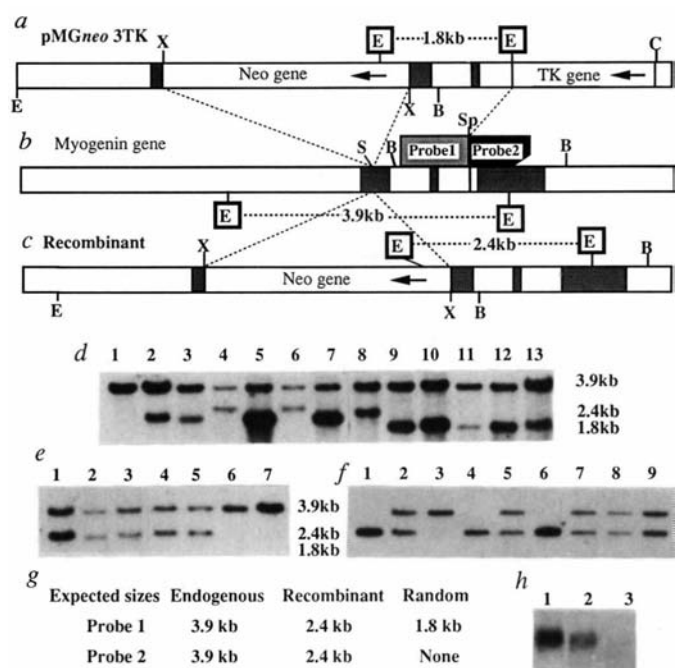
including myogenic cells disappear at 14.5 days p.c., immediately after the stage that myotube formation occurs (Fig. 2A, Bb, Cb). Accretion of fluid progresses rapidly from the body wall towards the head and limbs and finally the skull, the dorsal thorax and pelvis could be seen through the skin (Fig. 2A). In limbs of the mutants, cell masses stained with eosin were seen in the corresponding areas (Fig. 2D, b) where there were differentiated muscles in wild-type embryos (Fig. 2D, a). Although mature multinucleated myotubes were not detectable in cell masses, it is possible that severely disorganized short myotubes were included in cell masses, although most cells appeared to be mononucleate. To test whether these mononucleate cells have the properties of myogenic cells, we cultured cells freshly prepared from 14.5-days-p.c. forelimbs of mutant and wild-type embryos and characterized them as follows. (1) Cells from mutant embryos (about 4–5% of the cells on the dishes) differentiated to myotubes after 4 to 5 days in culture in differentiation medium (Fig. 4A, b and c), and many more myotubes were detected on dishes of mutant limbs compared with the numbers on dishes of wild-type limbs (Fig. 4A, a). This is probably because in the latter only a few myoblasts were retained as most myoblasts had already differentiated to myotubes at this stage *in utero*. From three independent experiments, we estimate that more than 10^4 myoblast cells

were present in one forelimb of a mutant embryo at 14.5 days p.c. Therefore, many myoblasts exist in limbs of mutant embryos, which are able to differentiate to myotubes on dishes in differentiation medium in the absence of myogenin. (2) This result was confirmed with fibroblasts prepared from mutant embryos, when ~1.5 to 2% of fibroblasts were successfully converted to myotubes by the transient transfection of *myoD* expression plasmids (Fig. 4C, b). These observations indicate that cells of the myogenic lineage in mutant limbs have the potential to differentiate to multinucleated myotubes, as seen in the axial skeleton, and it may be possible that some severely disorganized short myotubes are included in cell masses seen in mutant limbs.

Considering the differences in the extent of disorganization of the two muscle groups in the limb and axial skeleton, we propose the following possibilities. First, there may be an intrinsic difference in the muscle differentiation programmes of the two muscle groups¹⁰. It has been suggested that muscles in the axial skeleton and upper back muscles originate from myotomal myocytes in somites¹¹, whereas muscles in the latero-ventral body wall and limb are formed by cells that migrate from somites¹². Myotome cells start to differentiate as mononucleate myocytes *in situ* within the somite at 8.5 days p.c. to give rise to small spindle-like myotomal fibres¹³ before the initial expression of myogenin

FIG. 1 Disruption of the myogenin gene in mouse embryonic stem cells. a, Restriction map of targeting construct, consisting of a myogenin 3.5-kb *EcoRI*–*SpeI* genomic fragment containing exons 1 and 2. A *neo* cassette was inserted into exon 1 and a genomic fragment was flanked by a thymidine kinase (TK) cassette. b, Restriction map of the mouse genomic myogenin fragment. A 9-kb genomic DNA fragment encompassing the entire myogenin sequence is illustrated. Open boxes represent introns and flanking regions; filled boxes represent exons. The probes used for identification of allele-specific recombination are shown. c, Predicted structure of a mutated allele after homologous recombination. d, Initial screen of targeted myogenin clones by Southern blot hybridization analysis. The *EcoRI* 2.4-kb restriction fragments reveal the expected recombinant allele (lanes 4, 6 and 8). A DNA sample from untransfected cells is shown in lane 1. DNAs were digested with *EcoRI* and probed with probe 1. e, Confirmation of the targeted disruption. DNA samples from untransfected cells (lane 7), candidate recombinant clones (lanes 1–5), and a random integration clone (lane 6) were digested with *EcoRI* and probed with probe 2. f, Genotype of a littermate. Wild-type (lane 3), heterozygous mutant (lanes 2, 5, 7–9), and homozygous mutant (lanes 1, 4 and 6). DNAs were digested with *EcoRI* and probed with probe 1. g, Expected sizes of the *EcoRI* fragment detected by the two probes. Related *EcoRI* sites are boxed and the expected sizes are shown in the restriction maps. h, Northern blot patterns of wild type (lane 1), *MyoG*^{mt-1} heterozygous (lane 2), and *MyoG*^{mt-1} homozygous (lane 3) littermates. Poly(A)⁺ RNA was isolated from a whole embryo of 15.5 days p.c. and hybridized with the myogenin-specific probe. The amounts of RNA are normalized relative to the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Abbreviations: E, *EcoRI*; B, *BamHI*; S, *SacI*; X, *XhoI*; Sp, *SpeI*; C, *Clal*.

METHODS. The murine genomic myogenin DNA fragment was isolated from a BALB/c genomic library. A 3.5-kb genomic DNA fragment extending from an *EcoRI* site in the 5' upstream region to an *SpeI* site in intron 2 was subcloned into pBluescript vector for construction of a targeting vector. A double-stranded synthetic oligonucleotide (with the following sequence, which includes an *XhoI*: 5'-GTGAAGCTTCTCGAGT-3') was integrated into the *SacI* site in exon 1. The *neo* cassette isolated from pSTneo B (ref. 21) by *XhoI* digestion, was introduced into a synthetic *XhoI* site in exon 1 to disrupt the coding sequence of myogenin (pMyoGneo). For negative selection, pMyoGneo is flanked by a TK-cassette from pSHT at the 3' end. The resulting plasmid containing 2 kb of the myogenin sequence homologous at its 5' end and 1.5 kb at the 3' end was designated pMGneo 3TK. The unique *Clal* site in pMGneo 3TK allowed the plasmid to be linearized at the 3' end. ES cell line E14 (ref. 22) derived from a 129/Ola male mouse blastocyst was a gift from M. L. Hooper. Trypsinized ES cells (10^7) were suspended in 0.5 ml cold Hank's solution without Mg^{2+} and Ca^{2+} and then mixed with a targeting vector DNA at the concentration of $25 \mu\text{g ml}^{-1}$ in an electroporation cuvette, and electroporated at 200 V and 500 μF using Gene Pulser (Bio Rad) apparatus²³. After 2 days, neomycin (G418) at $400 \mu\text{g ml}^{-1}$



and gancyclovir (gift from Syntex Discovery R.) at 2×10^{-6} M were added to the culture to isolate the cells containing a targeted disruption by positive-negative selection, and surviving colonies were cloned after 7 d of selection. About 10 μg DNA was digested with *EcoRI*, fractionated by electrophoresis through 0.8% agarose gels, transferred to Hybond N nylon membrane (Amersham), and hybridized with probe 1 or 2. Transfer, hybridization and washing conditions followed the manufacturer's recommendations. Blastocysts were recovered from the mating B6C3F1 (F_1 of C57BL/6 and C3H/He) and C57BL/6 mice. ES-injected blastocysts were transplanted into uteri of pseudopregnant ICR mice. Chimeric mice were mated with BALB/c mice. Because of the genotypes determining the coat colour of the mice, 129/Ola (homozygous for whitebelly chinchilla, pink-eyed dilution; A^w/A^w , b/b , c^{ch}/c^{ch} , p/p) and BALB/c (albino; a/a , b/b , c/c), germ-line transmission of ES-derived genotype is recognizable by the light chinchilla coat colour of the animal. Poly(A)⁺ RNA was isolated by using a Quick Prep mRNA Purification Kit (Pharmacia) and electrophoresed, transferred and hybridized with a specific probe encoding the 3' 932 nucleotides of myogenin mRNA, as described previously²⁴.

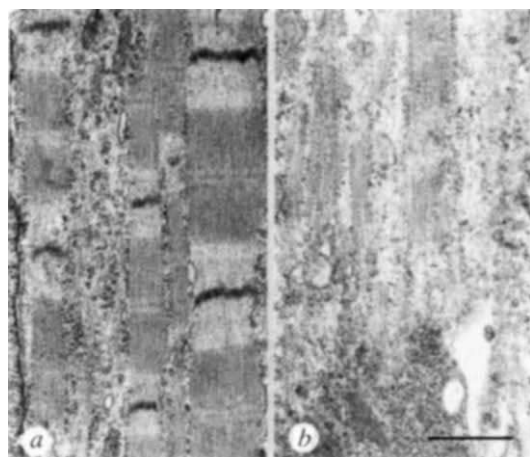


FIG. 3 Electron micrographs of intercostal muscles from wild-type and *MyoG^{mt-1}/MyoG^{mt-1}* embryos. Transmission electron microscopy was done on intercostal muscles from wild-type (a) and *MyoG^{mt-1}/MyoG^{mt-1}* (b) embryos at E14.5. Embryos were fixed in freshly prepared 4% paraformaldehyde, post-fixed in 1% O_3O_4 for 15 min, dehydrated and embedded in epoxyresin. Longitudinal sections were processed and viewed under a Hitachi electron microscope. Scale bar, 1 μ m.

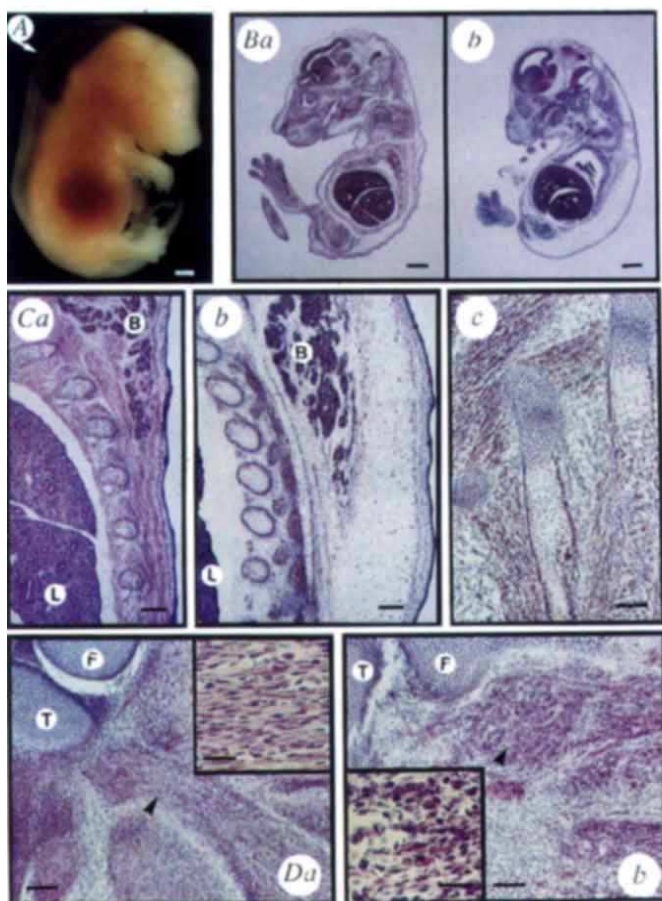


FIG. 2 Phenotypes of embryos from a $+/MyoG^{mt-1} \times +/MyoG^{mt-1}$ cross. E16.5 embryo of *MyoG^{mt-1}/MyoG^{mt-1}* (A). Arrow shows accretion of fluid under the skin. Parasagittal sections of E16.5 embryos of wild (B, a) and *MyoG^{mt-1}/MyoG^{mt-1}* (B, b). The intercostal muscles and body wall of E14.5 embryos of wild type (C, a) and *MyoG^{mt-1}/MyoG^{mt-1}* (C, b). Axial and intercostal muscles of an E14.5 embryo of *MyoG^{mt-1}/MyoG^{mt-1}* (C, c). L; Lung, B; Back muscle. The limbs of E14.5 embryos of wild type (D, a) and *MyoG^{mt-1}/MyoG^{mt-1}* (D, b). F; femur, T; tibia. The areas of the insets are indicated by arrowheads. In A and B scale bar is 1 mm; in C a and b, 200 μ m; in C c and D, 100 μ m; insets, 25 μ m. Embryos were fixed in paraformaldehyde, frozen-sectioned and stained with haematoxylin and eosin.

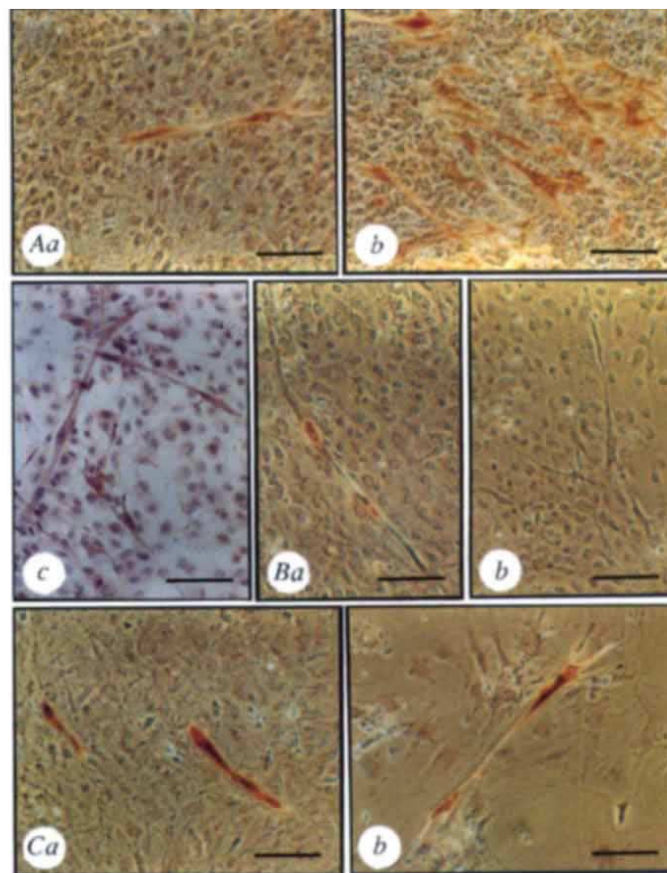


FIG. 4 Properties of cells prepared from the limbs of wild-type and *MyoG^{mt-1}/MyoG^{mt-1}* embryos. Cells prepared from wild-type embryos (A, a) and cells from *MyoG^{mt-1}/MyoG^{mt-1}* embryos (A, b) are stained with an anti-troponin T antibody²⁴; cells prepared from *MyoG^{mt-1}/MyoG^{mt-1}* embryos (A, c) are stained with haematoxylin and eosin; cells prepared from wild-type (B, a) and mutant (B, b) embryos are stained with an anti-myogenin antibody². Freshly prepared cells from limbs of E14.5 embryos were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (growing medium) for 2 days, followed by incubation for 4–5 days in DMEM with 2% horse serum (differentiation medium) to induce myogenic differentiation²⁵. Cells were fixed in 4% paraformaldehyde for 30 min, then in 0.25% Triton X-100 for 10 min, followed by two washings with phosphate-buffered saline. Immunostaining was carried out with an anti-troponin T or anti-myogenin antibody as described^{24,25}. Myotubes formed by forced expression of *myoD* in mouse primary fibroblasts prepared from wild-type (C, a) and *MyoG^{mt-1}/MyoG^{mt-1}* (C, b) embryos. Plasmid expressing *myoD* under the control of the human elongation factor-1 α promoter (pEFMD) was constructed by replacement of the chloramphenicol acetyl transferase (CAT) sequence of pEFCAT (ref. 26) with the mouse *myoD* cDNA sequence¹. About 10^5 cells were transfected with 10 μ g pEFMD. Cells were cultured in growth medium for 2 d, followed by incubation for 6 d in differentiation medium, and fixation and immunostaining with anti-troponin T antibody. Bar, 100 μ m.

protein¹⁴. On the other hand, the expression of myogenin is first detected at 11.5 days p.c. in forelimb buds and is abundantly expressed at 13 days p.c.⁹, the stage when terminal differentiation and myotube formation occurs, suggesting that the steps of terminal differentiation and myotube formation in muscles derived from migrating myogenic cells is more dependent on myogenin than that in myogenic cells of the myotomal lineage. Second, the growth stimulation to which cells of the two muscle lineages respond may be different. Inhibitory effects on muscle differentiation of growth factors such as FGF and TGF- β have been reported and growth signals that suppress or modulate the functions of myogenin and other members of myogenic bHLH proteins have been studied^{15–18}.

Given that some muscles form in mutant mice, that muscle differentiation takes place in cultured myoblasts of these mice, and that mutant fibroblasts will convert to myogenesis, we conclude that myotube formation can occur in the absence of myogenin. But myogenin is necessary for correct muscle formation and this includes the efficient formation of myotubes, the maturation of myotubes to mature muscle fibres, and probably the maintenance of the differentiated state. In addition, myogenin normally takes part in the terminal differentiation process from myoblasts to myotubes, because many myoblasts are retained as undifferentiated cells in the limbs of mutant embryos.

MyoD (ref. 19) and *myf5* (ref. 20) genes have been inactivated in mice by homologous recombination. Mice lacking *myoD* have normal skeletal muscle and the inactivation of *myf5* leads to abnormality of the ribs. From these results, a redundancy of functions of myogenic bHLH proteins has been suggested. We have shown that myogenin, a member of this family, is essential

for muscle development, and that other members of the myogenic gene family cannot compensate for the defect. The molecular function of myogenin in myogenesis remains to be elucidated in detail. □

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NMDA-dependent superoxide production and neurotoxicity

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NEURONAL injury resulting from acute brain insults and some neurodegenerative diseases implicates *N*-methyl-D-aspartate (NMDA) glutamate receptors^{1–4}. The fact that antioxidants reduce some types of brain damage suggests that oxygen radicals may have a role^{5–7}. It has been shown that mutations in Cu/Zn-superoxide dismutase (SOD), an enzyme catalysing superoxide (O_2^-) detoxification in the cell, are linked to a familial form of amyotrophic lateral sclerosis (ALS)⁴. Here we report that O_2^- is produced upon NMDA receptor stimulation in cultured cerebellar granule cells. Electron paramagnetic resonance was used to assess O_2^- production that was due in part to the release of arachidonic acid. Activation of kainic acid receptors, or voltage-sensitive Ca^{2+} channels, did not produce detectable O_2^- . We also find that the nitron DMPO (5,5-dimethyl pyrrolidine 1-oxide), used as a spin trap, is more efficient than the nitric oxide synthase inhibitor, L-*N*^G-nitroarginine, in reducing NMDA-induced neuronal death in these cultures.

In the presence of the short-lived O_2^- and hydroxyl ($\cdot OH$) radicals, the nitron DMPO yields the more stable spin adducts DMPO-OOH and DMPO-OH, respectively⁸. These nitroxides produce specific 12-lined ($a_N = 14.15$ G, $a_{H\beta} = 11.33$ G, $a_{H\gamma} = 1.37$ G) and 4-lined ($a_N = a_{H\beta} = 14.9$ G) EPR spectra, respectively (Fig. 1a, b).

The incubation medium (Fig. 1 legend) did not contain any

detectable spin adducts when incubated with or without cultured cells (CTR in Fig. 1c). After a 15-min incubation period with NMDA a prominent EPR signal was recorded that consisted of DMPO-OOH (Fig. 1c, filled circles) slightly contaminated with DMPO-OH (Fig. 1c, diamonds), which accounts for ~15% of the overall signal. This signal was abolished with (1) MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate), a non-competitive inhibitor of the NMDA receptor (Fig. 1c); (2) APV (2-amino-5-phosphonopentanoic acid), a competitive NMDA receptor antagonist, but not 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), a competitive inhibitor of the non-NMDA glutamate receptors (Fig. 1c), or (3) SOD (Fig. 1c). A signal with a major DMPO-OOH component was also detected with glutamate (Fig. 1d). This indicates that neurons behave as O_2^- generators upon NMDA receptor activation. SOD, which does not enter cells, suppresses the DMPO-OOH signal, indicating that the signal is due to O_2^- trapped by DMPO extracellularly. This does not exclude the possibility that some O_2^- is not detected because it remains inside the neurons. The spectral suppression of the DMPO-OH adduct by SOD suggests that it originates from intramolecular decomposition of DMPO-OOH⁸, rather than from genuine trapping of $\cdot OH$. In the absence of external Ca^{2+} (EGTA, ethylene glycol tetraacetic acid, 0.5 mM), no NMDA-generated signal is detected (Fig. 1c).

Interestingly, L-Narg (L-*N*^G-nitroarginine), a competitive inhibitor of nitric oxide synthase (NOS), did not modify the spectrum obtained with NMDA (Fig. 1d). Kainate, an agonist of the non-NMDA glutamate receptors, or KCl were inactive (Fig. 1c). As NMDA receptors stimulate arachidonic acid release in cultured neurons⁹, arachidonic acid was tested and found to generate the DMPO-OOH adduct (Fig. 1d), suggesting that it could be one of the precursors of O_2^- generated after NMDA receptor activation. This hypothesis was confirmed using mepacrine, a potent (although not very specific) phospholipase A₂ inhibitor, which inhibited the NMDA-induced signal (the concentration giving 50% inhibition, IC₅₀ was 20 μM ; Fig.

1d). As both indomethacin (10 μ M), a cyclooxygenase inhibitor, and *nor*-dihydroguaiaretic acid (1 μ M), a lipoxygenase inhibitor, nonspecifically inhibited the DMPO-OOH adduct generated by the xanthine-xanthine oxidase couple (XA-XO; 250 μ M, 20 mU ml⁻¹) in the presence of DMPO, we were unable to assess the role of these enzymes in NMDA-induced O₂⁻ production.

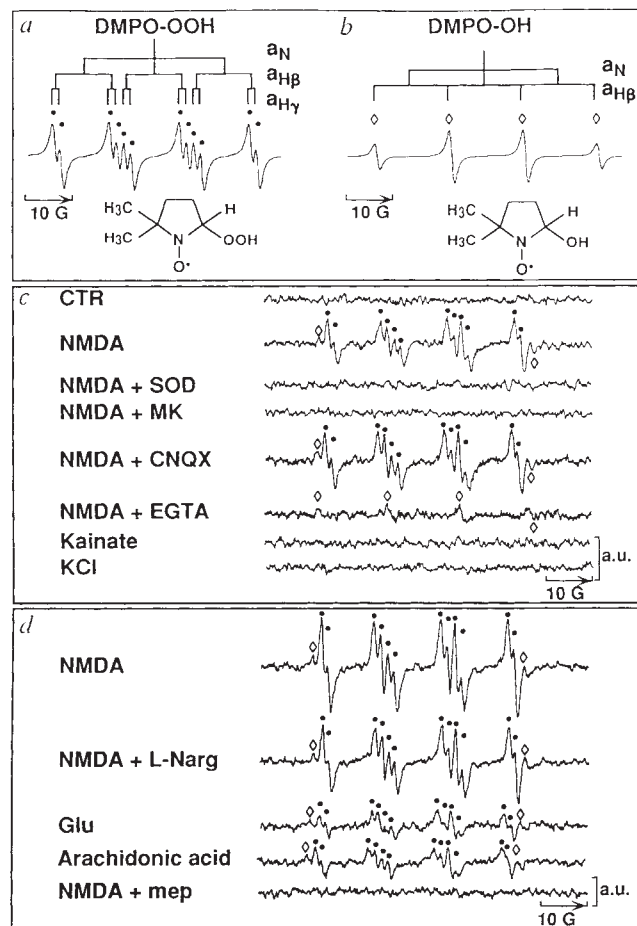


FIG. 1 EPR evidence that NMDA, glutamate and arachidonic acid, but not kainate and KCl, induce O₂⁻ formation in cerebellar granule cells. **a**, Computer-simulated EPR 12-lined spectrum and chemical structure of the superoxide DMPO spin adduct DMPO-OOH. **b**, Computer-simulated EPR 4-lined spectrum and chemical structure of the hydroxyl DMPO spin adduct DMPO-OH. **c**, **d**, Spectra are representative of at least three separate experiments. Control experiments with DMPO using XA-XO (250 μ M, 20 mU ml⁻¹) $\pm$ 20 μ M allopurinol (an inhibitor of XO) showed that none of the drugs interfere with the kinetics of either DMPO-OOH formation or breakdown.

METHODS. Mouse cerebellar granule cell (8×10^5 per well) cultures were prepared as described²⁵ and used at 10 days *in vitro*. After a 15-min wash with Locke's solution²⁶ at 37 $^{\circ}$ C and in the dark, cells were stimulated in the presence of the spin trap DMPO (100 mM), the iron-chelating agent DTPA (diethylenetriamine penta-acetic acid; 500 μ M) to prevent iron-dependent [•]OH formation, glycine (3 μ M) to activate the NMDA receptor fully and in the absence of Mg²⁺ to avoid any NMDA-associated channel blockade. The incubation medium was transferred 15 min (**c**) or 20 min (**d**) later into a standard quartz flat cell which was placed within the cavity of a Bruker ECS 106 EPR spectrometer equipped with 100 kHz modulation. Spectra were acquired at 25 $^{\circ}$ C within 45 s (modulation amplitude 0.9 G; microwave power 10 mW; receiver gain 5×10^5 ; time constant 163 ms; sweep rate 1.67 G s⁻¹). Spectral intensities are quoted using the same arbitrary units (a.u.). Drug concentrations were CNQX, 30 μ M; glutamate (Glu), 3 mM; KCl, 50 mM; mepacrine (mep), 300 μ M; MK-801 (MK), 1 μ M; SOD, 500 mU ml⁻¹; all others 100 μ M.

The NMDA effect was concentration-dependent (Fig. 2a); it appeared after a 10-min latency period (Fig. 2b), increased between 15 and 25 min, and then returned to basal level after 40 min incubation with neurons. Considering that DMPO-OOH is prone to fast enzymatic bioreduction to DMPO-OH in cellular systems¹⁰ and that there was a slow increase in DMPO-OH (Fig. 2b), we conclude that the decrease in DMPO-OOH signal observed after 30 min reflects a slowing in O₂⁻ generation. With 20 mU XO ml⁻¹ and 250 μ M XA, we obtained a DMPO-OOH signal comparable to that recorded in the presence of 300 μ M NMDA (Fig. 2c).

After a 30-min incubation period with NMDA or with the XA-XO system, the kinetics of neuronal death were comparable (Fig. 3a), indicating that O₂⁻ may be involved in NMDA-induced neuronal death. Indeed, DMPO was able to block (IC₅₀ $\approx$ 30 mM) this neuronal death (Fig. 3b). A similar blockade was obtained with two other nitrones, 3,3,5,5-tetramethyl pyrroline 1-oxide (TMPO; IC₅₀ $\approx$ 3 mM) and α -phenyl *N*-tert-butyl-nitron (PBN; IC₅₀ $\approx$ 10 mM) (Fig. 3b). The higher efficacy of TMPO (Fig. 3b), a more substituted DMPO derivative, could be due to its higher liposolubility¹¹ and therefore higher cell penetration. The absence of protection of the NMDA-induced neurotoxicity with SOD $\pm$ catalase (both tested from 100–700 U ml⁻¹) probably reflects the inability of these enzymes to penetrate cells, in contrast to DMPO¹². Whole-cell recordings of neurons indicate that NMDA currents are not affected by DMPO or PBN. L-Narg, which completely inhibited NMDA-induced NO formation in cerebellar neurons (Fig. 3c), had a modest effect on NMDA-induced neuronal death (Fig. 3d), in contrast to that of DMPO (Fig. 3d).

We have described the generation of superoxide upon NMDA

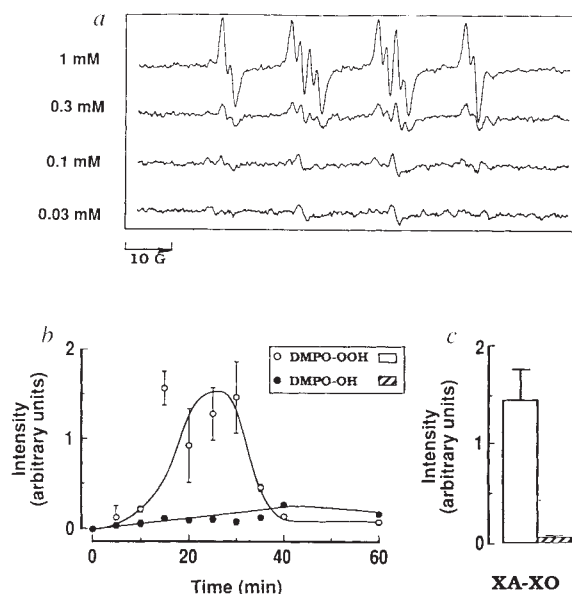


FIG. 2 Concentration response and time course of DMPO-OOH and DMPO-OH accumulation under NMDA stimulation of cerebellar granule cells in the presence of DMPO (100 mM) and DTPA (500 μ M). **a**, NMDA concentration-response scans from one representative experiment. **b**, Each point of the curve represents the mean $\pm$ s.e.m. of at least three determinations, each done on different culture wells in two different cultures. NMDA concentration, 100 μ M. **c**, Intensity $\pm$ s.e.m. ($n=4$) of DMPO-OOH and DMPO-OH EPR signals recorded at 2 min using XA-XO (250 μ M, 20 mU ml⁻¹) superoxide generator in DMPO-supplemented medium without cells.

METHODS. Cerebellar granule cells used at 10 days *in vitro* were exposed to NMDA (100 μ M) for the indicated times. The concentrations of the spin adducts were determined by double integration of the low-field doublet (DMPO-OOH) or singlet (DMPO-OH) of the EPR spectrum. Other methods as described in Fig. 1, legend.

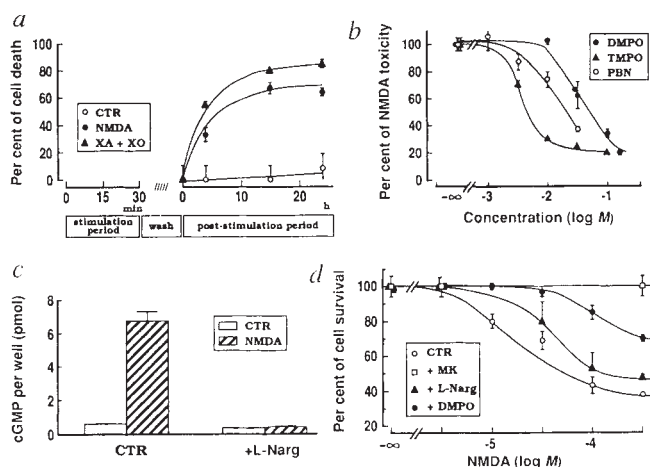


FIG. 3 Neuronal death produced by 30 min stimulation with XA-XO or NMDA. Kinetics and neuroprotection by L-Narg and nitrones. *a*, Post-stimulation kinetics of the cytotoxicity induced by 30-min stimulation with XA-XO (250 μ M, 3 mM ml^{-1}) or NMDA (100 μ M). *b*, Neuroprotective effects of increasing concentrations of DMPO, TMPO and PBN on the neuronal death induced by NMDA (100 μ M) and measured 24 h subsequent to stimulation. *c*, NMDA-dependent cGMP production is blocked by L-Narg (100 μ M). *d*, Effect of MK-801 (1 μ M), L-Narg (100 μ M) and DMPO (100 mM) on neuronal death induced by increasing concentrations of NMDA and measured 24 h after stimulation. Results are means $\pm$ s.e.m. of triplicate measurements and representative of at least three different experiments.

METHODS. Neurotoxicity experiments were done on cerebellar granule cells at 10 days *in vitro* under sterile conditions at 37 $^{\circ}\text{C}$ and in the dark, as described^{26,27}. Briefly, culture medium was removed and stored, culture wells were washed twice in Locke's solution (Fig. 1 legend) for 15 min and incubated at 37 $^{\circ}\text{C}$ for 30 min in 0.5 ml Locke's solution (Mg^{2+} -free) containing glycine (3 μ M) and the substances to be tested (stimulation period). Cells were washed twice with Locke's solution and incubated again with the initial stored medium at 37 $^{\circ}\text{C}$ for different periods (that is, the post-stimulation period), the remaining living cells were surveyed by phase contrast microscopy then treated by the fluorescein-diacetate method²⁸. The percentage of cell death or cell survival (*a*, *d*) was calculated relative to the control without agonist. The per cent of NMDA toxicity (*b*) was calculated relative to (control - NMDA)/control = 100% of toxicity. cGMP experiments (*c*) were done for 5 min and measurements made as described²⁹. Note that in our conditions, XO is toxic only in the presence of XA.

receptor stimulation. The production of oxygen radicals by glutamate receptors has long been presumed on the basis of two NMDA-induced effects: the increase of intracellular calcium^{13,14} and the release of arachidonic acid⁹, both of which can generate oxygen radicals¹⁵, but this has not been directly demonstrated before. Our results show that NMDA induces a calcium- and arachidonate-dependent O_2^- formation in neuronal culture (Fig. 1*d*). Moreover, K^+ , which does not stimulate arachidonic acid release in neurons⁹, does not induce O_2^- production (Fig. 1*c*).

NOS can produce O_2^- when L-arginine concentrations are low^{16,17}. NOS is certainly not the main pathway in O_2^- production under NMDA stimulation because (1) neurons are not depleted of L-arginine, as indicated by their high capacity to produce NO (Fig. 3*c*), and (2) L-Narg did not suppress O_2^- production (Fig. 1*d*).

In cerebellar granular cells, trapping O_2^- seems to be more efficient than blocking NO production to reduce NMDA-induced neuronal death. Blocking NO formation prevents glutamate neurotoxicity in some but not all *in vitro* and *in vivo* studies¹⁸⁻²². Neuronal susceptibility to NO- or O_2^- -induced damage could depend on the nature of the neurons and on the environmental conditions. Cerebellar granule cells, known to contain high levels of NOS²³, could be more resistant than other neurons to NO-induced cell death²⁴. □

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The synaptic vesicle protein synaptotagmin promotes formation of filopodia in fibroblasts

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NEURONAL filopodia are actin-rich cytoplasmic extensions that are involved in motility and recognition in growth cones and maturing axonal endings. A detailed understanding of neuronal growth will depend on clarification of the membrane fusion events occurring during filopodial extension. The synaptic vesicle protein synaptotagmin seems to be intimately involved in exocytotic membrane fusion¹⁻³. Here we show that fibroblast cell lines transfected with synaptotagmin form long, highly branched, actin-rich filopodial processes, with the expressed synaptotagmin being incorporated into the plasma membrane. In contrast, cell lines expressing either of two other synaptic vesicle proteins, SV2 or synaptophysin, generate only rudimentary processes, and, like neurons, sort SV2 and synaptophysin to small intracellular vesicles. As presynaptic calcium entry regulates synaptic vesicle fusion, our results indicate that synaptotagmin might link neuronal activity with synaptic growth.

Stably transfected fibroblastic CHO (Chinese hamster ovary) cells expressing synaptotagmin elaborate many long, highly branched processes (Fig. 1*a,b*). In contrast, cells transfected with the vector alone had fewer and shorter processes (Fig. 1*c*).

Approximately twenty stable cell lines were originally isolated. Cells from each of the independently derived lines grew long, complex filopodia. Three cell lines with the highest synaptotagmin expression were selected for further study. To quantify the morphological changes, we measured the number of processes per cell, their length and extent of branching, in three independently derived synaptotagmin cell lines and in three control cell lines (Fig. 2). Expression of synaptotagmin, but not SV2 (refs 4,5) or synaptophysin, dramatically enhances process growth. Western blot analysis confirmed expression of the transfected genes, revealing an appropriately sized protein in synaptotagmin, SV2 and synaptophysin cell lines, but not in cells transfected with the vector alone (Fig. 3).

To investigate the processes induced by synaptotagmin further, we double-stained cells for synaptotagmin and filamentous actin (Fig. 4e,f). The synaptotagmin-induced processes contained actin cables, like filopodia from untransfected cells, or from neurons. In many cases synaptotagmin appeared to be concentrated at the tips of the microfilaments (Fig. 4e). To follow the dynamics of filopodial formation, and to ensure that

the processes were not remnants of retracted cell bodies, we studied filopodial formation and retraction using video-enhanced phase microscopy. Most often, processes advanced from and were engulfed by the cell's leading edge. We also observed filopodial retraction, but saw no processes remaining after migration or contraction of the cell body.

The distribution of SV2 in transfected fibroblasts appears to be similar to that of synaptophysin (Fig. 4b,d; refs 4, 5, 16–20). Immunofluorescent staining revealed a punctate cytoplasmic pattern (Fig. 4d). In contrast, significant amounts of transfected synaptotagmin accumulated in the plasma membrane and filopodia (Figs 1, 4). To confirm synaptotagmin's incorporation into the plasma membrane, we used an antibody that recognizes the extracellular, or luminal, domain of synaptotagmin⁶. Plasma membrane staining was clear when non-permeabilized cells were incubated with the antiserum (Fig. 4g). No staining was seen in parallel cultures incubated with an antibody that recognizes a cytoplasmic determinant.

The presence of synaptotagmin in the plasma membrane is intriguing, because most light immunocytochemical studies

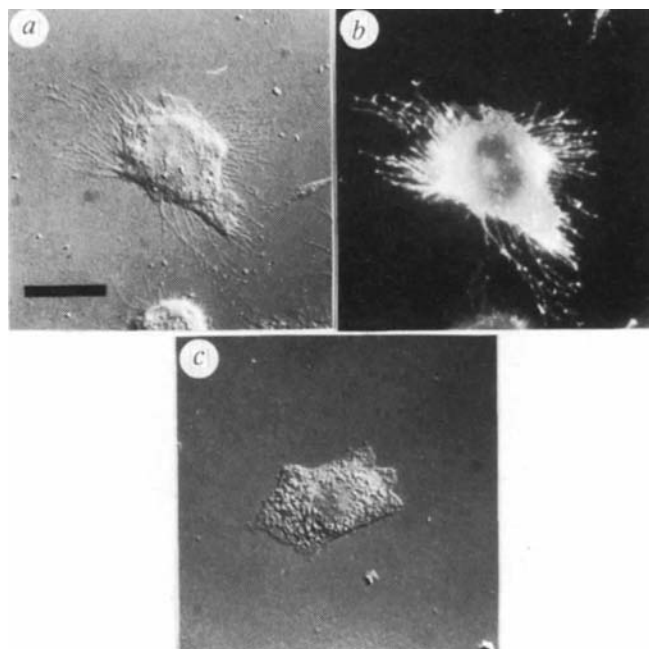
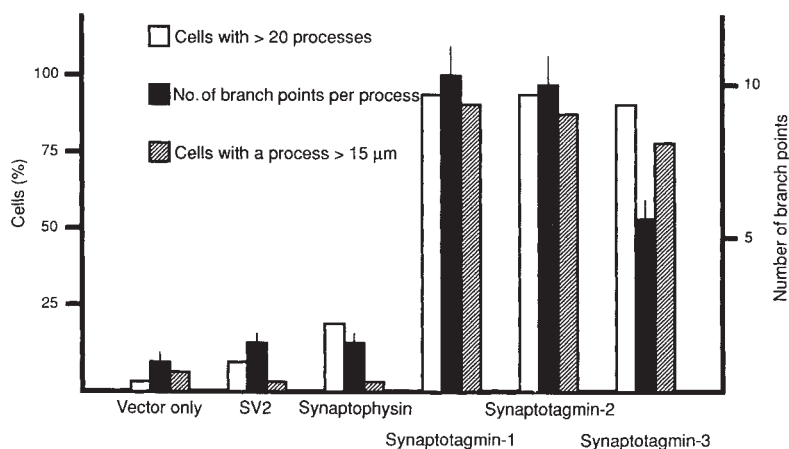


FIG. 1 *a*, Nomarski imaging of cells transfected with the gene for synaptotagmin demonstrates elaborate filopodial processes. *b*, These processes contain the synaptotagmin protein, shown by immunofluorescence using an antibody specific to synaptotagmin. *c*, Cells transfected with vector alone display few, short filopodia. Scale bar, 10 μ m.

METHODS. A cDNA encoding synaptotagmin was amplified from a rat brain cDNA library by PCR. The synaptotagmin clone was sequenced and two changes were found from the predicted amino-acid sequence of the previously published¹ rat cDNA: glutamic acid at position 188 was changed to aspartic acid (E188D) and D374 was altered to G. It is unlikely that these substitutions are PCR-induced errors because both are conserved in the human and *Drosophila* sequences³⁰. The synaptotagmin cDNA was subcloned into the expression vector pEE14, which contains the glutamine synthetase selectable marker gene³¹, and the CMV promoter³². CHO-K1 cells were transfected using lipofectin (from BRL) and stable transformants were selected. Lines were subcloned until 100% of the cells expressed synaptotagmin by immunocytochemistry using alkaline phosphatase detection. The synaptotagmin clone was also expressed in a second vector, pNL³³, and similar results were obtained. For immunofluorescence analysis, cells were grown overnight on poly-L-lysine-coated coverslips, fixed in 4% paraformaldehyde, permeabilized in a buffer containing 0.3% Triton X100 and incubated with monoclonal antibody 48 (ref. 7), which is specific for synaptotagmin. An identical staining pattern was seen using a rabbit polyclonal anti-synaptotagmin antiserum⁶.

FIG. 2 Quantification of the morphological effect of synaptotagmin expression reveals that three independently derived synaptotagmin expressing cell lines have a larger number of longer, more highly branched filopodia than cells transfected with vector alone, or with the unrelated synaptic vesicle proteins SV2 and synaptophysin.

METHODS. Synaptophysin-expressing clones were obtained using a synaptophysin cDNA in the vector pEE14 as for Fig. 1. The vector CDM8 was used to express SV2 (ref. 13). Cells were labelled as for Fig. 1, using monoclonal antibodies against SV2 (ref. 34) and synaptophysin (SY38; Boehringer–Mannheim). All measurements were made on cells plated in parallel. For the synaptic vesicle protein-expressing cells, only cells clearly expressing the transfected protein were selected for quantification. The extent of branching was determined by counting the number of branch points on the most branched process.



have failed to find notable synaptotagmin in neuronal plasma membrane⁶. Immunocytochemistry in the electron microscope⁷ does show some plasma membrane labelling, however, and gradient fractionation reveals a significant amount of synaptotagmin (but not synaptophysin) in purified adrenal chromaffin cell plasma membranes⁸. Expression in a foreign system may be emphasizing an important targeting difference between neuronal synaptotagmin and other synaptic vesicle proteins.

Differences in the levels of protein expression are unlikely to account for the contrasting distributions because the cells produce similar amounts of all three proteins (Fig. 3). Induction of extensive cellular processes is itself also unlikely to account for the plasma membrane accumulation of synaptotagmin, because cells expressing both synaptotagmin and SV2, both synaptotagmin and synaptophysin, or all three proteins, fail to accumulate SV2 or synaptophysin in the plasma membrane (M. B. F. *et al.*, manuscript in preparation). The presence of synaptotagmin in the plasma membrane and in filopodia is not specific to CHO cells. Similar expression patterns were observed in transient expression experiments using COS fibroblasts and undifferentiated ND7(ref. 9) (Fig. 4h) and NG108-15 neuroblastoma cell lines (data not shown).

By expressing the synaptic vesicle protein synaptotagmin in non-neuronal cells, we may have uncovered an unsuspected capacity of the protein to influence cell morphology. We do not know how synaptotagmin enhances filopodial growth in transfected fibroblasts, but it is likely that the synaptic vesicle protein interacts with the machinery that mediates normal fibroblast process outgrowth. In neurons, synaptotagmin binds the presynaptic plasma membrane proteins neurexin¹⁰⁻¹² and syntaxin¹³⁻¹⁶. The extracellular domains of the neurexins contain repeated sequences homologous to laminin A, the *Drosophila* neurological mutant *slit*, and agrin, all proteins important for axon guidance and synaptogenesis. Synaptotagmin may alter cellular morphology by interacting with similar proteins in fibroblasts. Of course, our observations in CHO fibroblasts

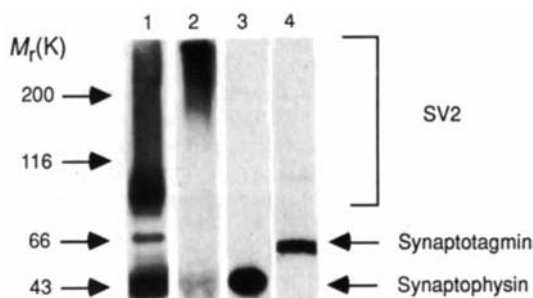


FIG. 3 Western blot analysis demonstrates comparable expression of the transgenes. SV2- (lane 2), synaptophysin- (lane 3) and synaptotagmin- (lane 4) expressing cell lines were immunoblotted and reacted with appropriate SV2 (ref. 34), synaptophysin (SY38) or synaptotagmin (mAb 48; ref. 7) antibodies. The SV2 protein is more highly glycosylated in transfected fibroblasts than in rat brain, but is similar to SV2 found in PC12 cells. Immunoblot of an equal amount of total rat brain homogenate protein incubated with all three antibodies simultaneously is shown in lane 1 for comparison. M_r markers are shown on the left.

METHODS. Cells were homogenized, centrifuged for at least 15 h at 48K in a SW50.1 rotor at 4 °C through a 10–50% sucrose gradient and 17 separate fractions were immunoblotted¹⁸; synaptic vesicle proteins were detected with ¹²⁵I-labelled goat anti-mouse immunoglobulin. An autoradiograph of the peak fraction of ~ 1.14 g ml⁻¹ is shown. Gradient centrifugation was necessary as the levels of transfected protein were too low to be detected reliably in the total cellular homogenate. Transfected cell lines were enriched for the synaptic vesicle proteins ~ 20-fold by gradient fractionation. Equal amounts of protein were loaded in each lane.

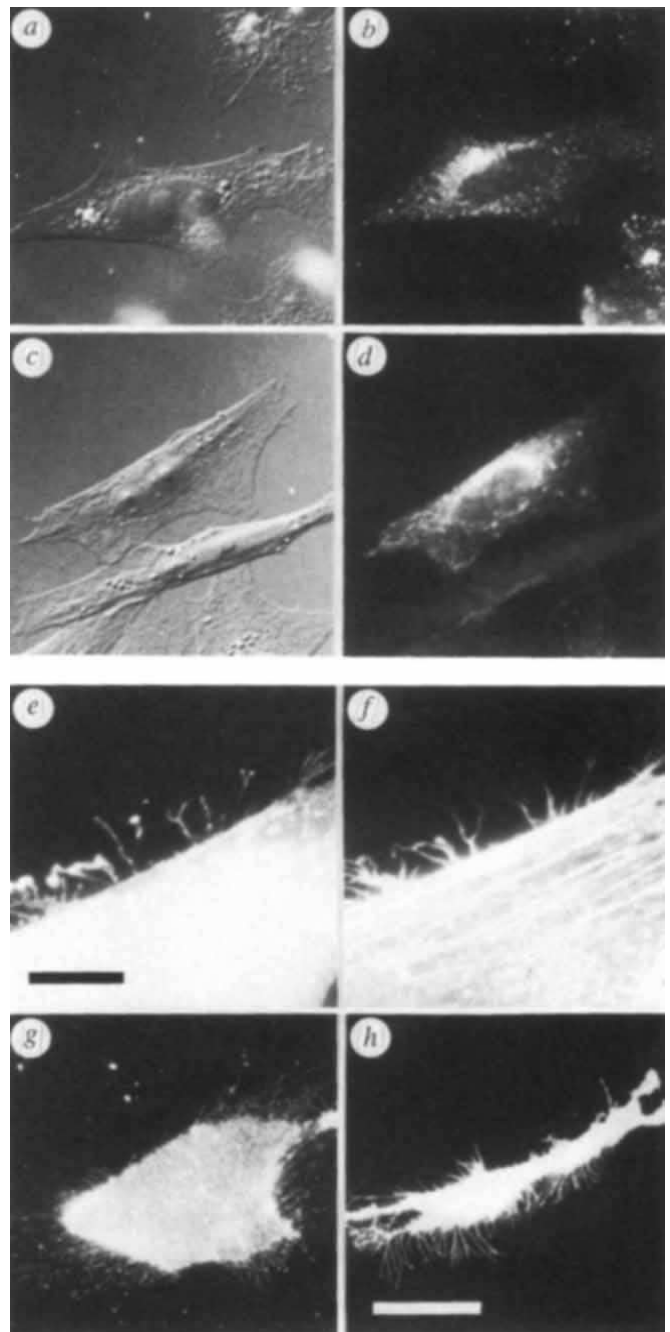


FIG. 4 Nomarski imaging of a, synaptophysin- and c, SV2-expressing CHO cells shows few filopodia. b, Immunofluorescent detection of synaptophysin reveals a punctate cytoplasmic staining. d, An antibody to SV2 shows a similar pattern. e and f, Immunofluorescent detection of synaptotagmin (e) and actin (f) in the same cells shows that synaptotagmin-induced processes contain both the synaptotagmin protein (e) and actin (f). g, Incubating nonpermeabilized cells with an antiserum recognizing the extracellular domain of synaptotagmin demonstrates the presence of synaptotagmin in the plasma membrane. h, Undifferentiated neuroblastoma (ND7) cells also accumulate transfected synaptotagmin in the plasma membrane and filopodia. Scale bars are the same as in Fig. 1, except in e, f, 5 µm, and in h, 50 µm.

METHODS. Cells were labelled for standard immunofluorescence as in Fig. 1. Cell-surface expression was determined by incubating unfixed cells in ectopeptide antiserum for one hour at 4 °C (g). Cells were then fixed and processed for immunofluorescence. Identical experiments were done in parallel using monoclonal antibody 48 which recognizes a cytoplasmic synaptotagmin epitope; no staining was seen. Actin was visualized using rhodamine-conjugated phalloidin (Molecular Probes). Transient transfection was carried out using lipofectin (BRL).

should be interpreted with caution. Neuron-specific post-translational modifications such as glycosylation and phosphorylation may modulate synaptotagmin function in the nervous system. CHO cells expressing synaptophysin¹⁷⁻²¹ or SV2 (M. B. F. *et al.*, manuscript in preparation) do not sort the proteins to synaptic vesicles; the formation of this specialized organelle and normal synaptic vesicle protein function may require the coexpression of other synaptic vesicle proteins, or additional neuronal proteins like the syntaxins and neurexins.

The cytoplasmic domain of synaptotagmin contains two copies of a region similar to the C2 domain of the phospholipid-dependent protein kinase C, and recombinant synaptotagmin binds acidic phospholipids. This attribute has led to speculation that synaptotagmin might mediate exocytotic vesicle fusion¹⁻³. One possible model of synaptotagmin action in transfected fibroblasts incorporates both the protein's filopodial concentration and its phospholipid binding capacity. Synaptotagmin-

containing vesicles are targeted to the growing fibroblast process. Here, interacting with one or more endogenous proteins, synaptotagmin mediates phospholipid interactions leading to vesicle fusion and process growth. Many synaptic vesicle proteins, including synaptotagmin, are expressed during neurite outgrowth²²⁻²⁶ in organelles that fuse with the plasma membrane^{27,28}. Post-mitotic migrating cells also express syntaxin, which binds synaptotagmin, and has homology to the neurite outgrowth-promoting region of the laminin B1 chain¹⁴. Besides a role in neuronal development, synaptotagmin could also participate in synaptic remodelling in the mature nervous system. Many models of synaptic plasticity in learning and memory postulate a connection between neuronal activity and synaptic growth or retraction²⁹. As synaptic vesicle fusion depends on presynaptic depolarization, synaptotagmin is ideally situated to participate in activity-dependent synaptic expansion. □

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Inhibition of Rab3B expression attenuates Ca²⁺-dependent exocytosis in rat anterior pituitary cells

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LOW-MOLECULAR-WEIGHT GTP-binding proteins (small G proteins) of the Rab family have been proposed to act as central regulators of vesicular traffic^{1,2}, and proteins of the Rab3 subfamily (Rab3A, B, C and D)³⁻⁶ are thought to be associated with membrane vesicles or granules undergoing exocytotic fusion with the plasma membrane⁷⁻¹⁰. Rab3A is highly expressed in brain¹¹, whereas Rab3B is the major form found in rat anterior pituitary gland. We report here that antisense oligonucleotides against Rab3B, introduced into pituitary cells using the whole-cell patch clamp technique, specifically and reversibly block expression of

Rab3B. We find that calcium-dependent exocytosis is inhibited without affecting endocytosis. Antisense oligonucleotides directed against Rab3A have no effect. Our results indicate that Rab3B is likely to be a key intracellular signalling molecule which can control exocytosis downstream of other calcium-dependent processes in anterior pituitary cells.

In endocrine cells, hormones are released mainly through calcium-regulated exocytosis by mechanisms that remain largely unknown¹². It has been suggested that Rab3 proteins regulate exocytotic vesicle movement in the cell and sorting to the plasma membrane^{13,14}. Using northern blotting analysis, we detected high levels of Rab3B but not Rab3A messenger RNA in the anterior pituitary gland (Fig. 1a). Two other forms of Rab3 (Rab3C and 3D; refs 4 and 6, respectively) have not been detected in neuroendocrine tissues and so are not considered here.

Because Rab proteins 3A and 3B have identical *M_r*s and isoelectric points, two-dimensional gel electrophoresis could not be used to determine their relative proportions in the anterior pituitary gland. We therefore exploited differences in nucleic acid sequence between the Rab3 family members to design deoxyribonucleotides in an antisense orientation to Rab3A and Rab3B mRNAs (Fig. 1b) which would block expression of these molecules. The antisense oligonucleotides PT3 and N63, which overlap the translation initiation site of Rab3A and Rab3B mRNAs respectively, inhibited the *in vitro* translation of the RNAs (Fig. 1c). In contrast, other oligonucleotides, whether sense or antisense, were inefficient. Because a specific anti-Rab3B antibody was not available to identify the protein, we used a polyclonal

antibody raised against purified Rab3A which crossreacts with Rab3B protein (Fig. 1d). N63 (antisense for Rab3B), but not PT3 (antisense for Rab3A), could reduce Rab3-like immunoreactivity on western blots of the *in vitro* translation products of pituitary poly(A)⁺ RNA (data not shown).

Rab3A and Rab3B expression was analysed in individual anterior pituitary cells loaded with different sense or antisense oligonucleotides with a patch pipette. The expression of Rab3 protein was checked by immunofluorescence using antibody against Rab3. Whereas a reproducible staining of non-loaded cells was observed (Fig. 2), the expression of immunoreactive Rab3 protein was strongly suppressed in cells stained 72 h after loading with N63 (Fig. 2b'). Cells injected with the Rab3A-specific antisense PT3 showed fluorescence similar to non-loaded cells (Fig. 2a'). With N61 (antisense complementary to a 3' non-coding part of the Rab3B mRNA; Fig. 1b), N62 (sense, in the coding Rab3B region; Fig. 1b) and PT5 (sense, in the coding Rab3A region; Fig. 1b), fluorescence was again similar to that seen in unloaded cells, demonstrating the specificity of the N63 antisense probe in suppressing Rab3B expression. We conclude that Rab3B is likely to be the dominant form present in anterior pituitary cells and that its biosynthesis can be efficiently blocked by the antisense N63.

To investigate a possible role of Rab3B protein in exocytosis, we used whole-cell recordings to monitor changes in membrane capacitance (C_m) during cytosol dialysis with solutions of various calcium concentrations, having previously injected the cell with specific oligonucleotides¹⁵ (see legend to Fig. 3). In control experiments, the increase in C_m related to exocytosis is induced by dialysis of the cytosol with 1 μ M calcium in the pipette solution, as shown previously¹⁶⁻¹⁸. The secretory response (ΔC_m)

measured as a change in C_m relative to resting C_m 7.5 min after the start of recording was increased by 30–40% (Fig. 3a, c and d; $n=34$). In contrast, cells loaded with oligonucleotide N63, showed a reduced secretory response 48 h after loading, with ΔC_m in the range of 8–18% (Fig. 3a, c and d; $n=5$). This diminution was not detectable 6 h after antisense loading, had developed only partially at 24 h, and was maintained for up to 72 h (Fig. 3c). It seems therefore that inhibition of Rab3B expression can strongly reduce the calcium-induced increase in C_m .

As calcium-induced changes in C_m express the sum of exocytosis and endocytosis, it is possible that a reduced secretory response in N63-treated cells could be due to an enhanced rate of endocytosis, even if the latter is mainly calcium-independent¹⁹. Therefore the effect of N63 loading was studied in cells in which the cytosol was dialysed with a low-calcium-containing pipette solution ($\sim 10^{-8}$ M). Under these conditions, exocytosis is blocked and a residual decrease in C_m consistent with net retrieval of membrane (endocytosis) can be monitored²⁰. Figure 3b shows that loading of oligonucleotide N63 into pituitary cells does not affect membrane retrieval. In control cells, ΔC_m measured 5 min after entering the whole-cell configuration was $-12 \pm 3\%$ ($n=10$), whereas in cells loaded for 48 h with N63 it was $-16 \pm 2\%$ ($n=9$). Thus the specific blocking of Rab3B expression does not modify endocytosis, although calcium-induced exocytosis is decreased. This can be compared with observations^{10,21} showing that a peptide corresponding to the conserved Rab3 effector domain both stimulates exocytosis and blocks vesicular transport. However, Rab3 *in vivo* may not play a role in early endocytosis events; it has been proposed that these are controlled by Rab4 (ref. 22) and Rab5 proteins^{23,24}.

The reduction in calcium-induced exocytosis parallels the dis-

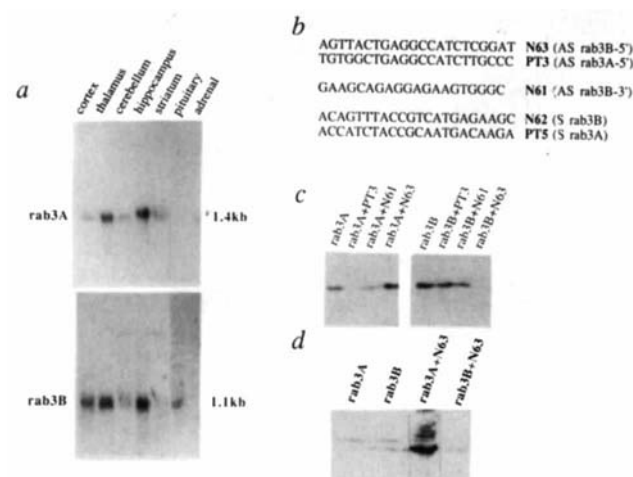
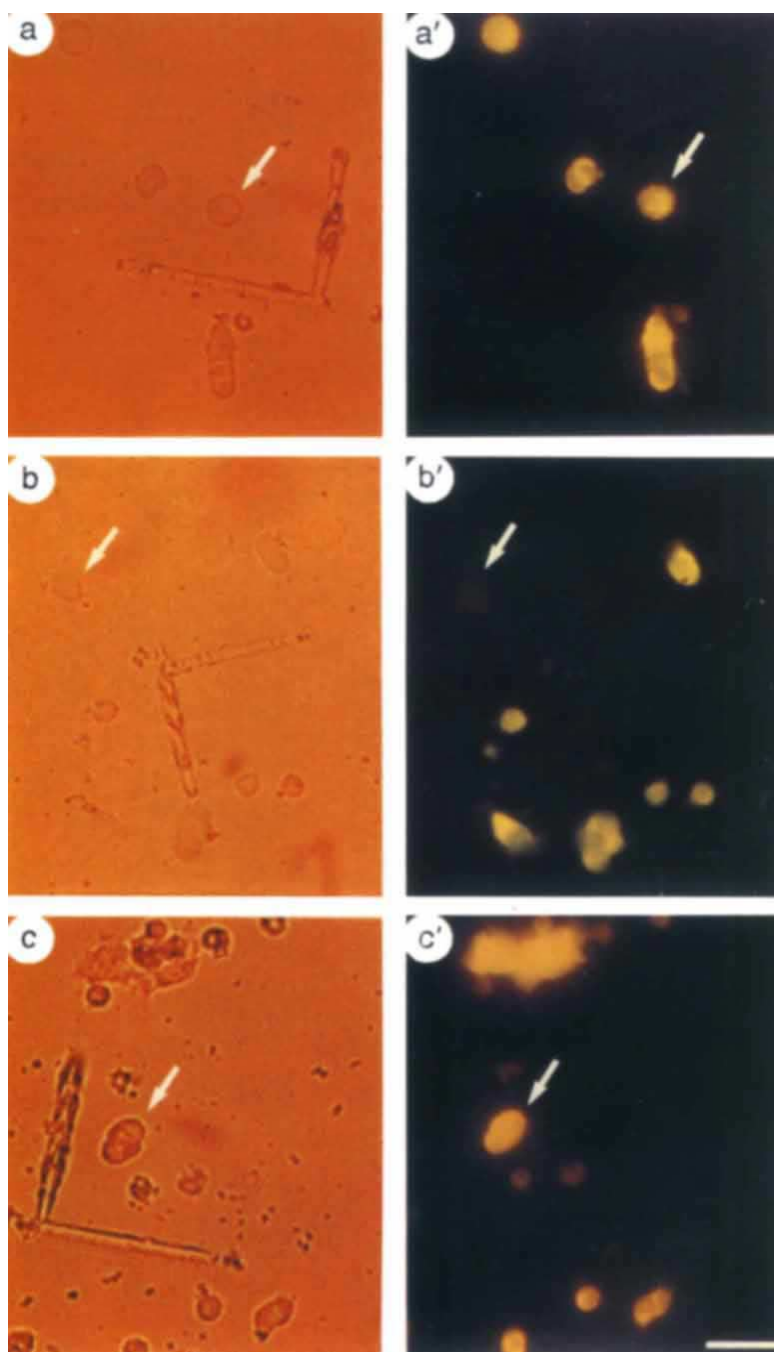


FIG. 1 Expression of proteins Rab3A and Rab3B in the anterior pituitary gland and the effect of specific antisense oligonucleotides. **a**, Northern blot analysis of Rab3A and Rab3B transcripts in neuroendocrine tissues. Top, hybridization with the rat Rab3A probe (autoradiogram exposed for 8 h); bottom, hybridization with the rat Rab3B probe (autoradiogram exposed for 4 d). **b**, Sequences of antisense (AS) and sense (S) oligonucleotides, oriented 5' to 3'. N63 and PT3 overlap the translation initiation codon of Rab3B and Rab3A respectively. N61 is complementary to a region of the 3' untranslated part of Rab3B mRNA. N62 and PT5 correspond to the nucleotides 188 to 208, relative to the first ATG in the coding region of Rab3B and Rab3A respectively. **c**, Autoradiograms of 12% SDS-polyacrylamide gels showing *in vitro*-translated Rab3A and Rab3B proteins (corresponding to about 1 mg total reticulocyte lysate protein) in the presence of the various antisense oligonucleotides. **d**, Western blot of a 15% SDS-PAGE of *in vitro*-translated Rab3A and Rab3B proteins labelled with the anti-Rab3A/B antibody. Loading was 1 mg (two left-hand lanes) or 10 mg (two right-hand lanes) of total reticulocyte lysate proteins. Note the cross-reaction of antibody with Rab3A and Rab3B proteins, as well as the almost complete translation inhibition by the N63 antisense. The lower band and upper bands correspond to isoprenylated and non-isoprenylated forms of the Rab3 proteins, respectively (P.V., F. Darchen and J. Mallet, manuscript in preparation).

METHODS. For northern blot analysis, 10 μ g total RNA extracted from various neuroendocrine organs were electrophoresed on formaldehyde-agarose gels, blotted on Hybond C-extra nitrocellulose membranes (Amersham) and successively hybridized with Rab3B and Rab3A ³²P-labelled cDNA probes prepared according to standard techniques. *In vitro* translations, either with or without antisense and sense oligonucleotides, were performed in rabbit reticulocyte lysate (Promega) with 10 μ g ml⁻¹ (final concentration) of Rab3A and Rab3B RNAs transcribed from T3 promoter in Bluescript plasmid. Translated proteins were labelled by incorporation of ³⁵S-methionine (Amersham), except before western blotting. All procedures followed the manufacturer's protocol. Translation products were denatured in Laemmli buffer at 95 °C for 2 min and size-fractionated on 12% or 15% acrylamide gels with 0.1% SDS. For western blotting, gels were electrotransferred on nitrocellulose membranes, stained with Ponceau red, preincubated and then incubated with anti-Rab3A/B antibody (1/1,000) for 12 h. Antibody labelling was revealed with ¹²⁵I-labelled protein A (Amersham) according to standard procedures.

FIG. 2 Immunocytochemical detection of Rab3 proteins in cultured rat anterior pituitary cells. Cells were photographed sequentially to visualize a mark scratched in Petri dishes for the identification of loaded cells (injected with about 50,000 molecules of DNA antisense) and fluorescein isothiocyanate (FITC)-IgG labelling of Rab3. **a**, Phase-contrast image showing a cell 72 h after whole-cell recording and loading with oligonucleotide PT3 (arrow). **a'**, FITC fluorescence image of the same cells as in **a** showing similar staining among loaded (arrow) and non-loaded cells. **b** and **c**, Phase contrast images showing cells after whole-cell recording and loading with oligonucleotide N63 (arrows). **b'** and **c'**, FITC fluorescence images of cells in **b** and **c** injected with oligonucleotide N63 and observed 72 h or 110 h afterwards, respectively. Immunofluorescence intensity was reduced only in the cell loaded for 72 h with N63 (**b'**). Note also that the nonspecific fluorescent signal of the debris can be compared to the immunofluorescence of the antisense-loaded cell shown in **b'**.

METHODS. Pituitary cells were isolated from the pars distalis of the rat pituitary gland. Glands were enzymatically dispersed using collagenase, and a lactotroph-rich population was produced by separation of cells on a discontinuous Percoll (Pharmacia) density gradient³⁰. Cells were cultured for 3–7 d at 36 °C in a water-saturated 5% CO₂ atmosphere before loading with oligonucleotides and cell membrane capacitance measurements. No difference was detected in the properties of cells in relation to time in culture. Indirect immunofluorescence staining was carried out as described⁸. Briefly, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 30 min, washed with PBS, incubated with 10 mM NH₄Cl, washed, and permeabilized for 1 h with 0.05% saponin/0.2% bovine serum albumin/PBS. They were then incubated with immunopurified anti-Rab3 antibody (1/40), washed, and incubated with fluorescein-conjugated anti-rabbit F(ab')₂ fragment (Sigma). All steps were done in a glycerol/PBS mixture at room temperature. Scale bar, 20 µm.

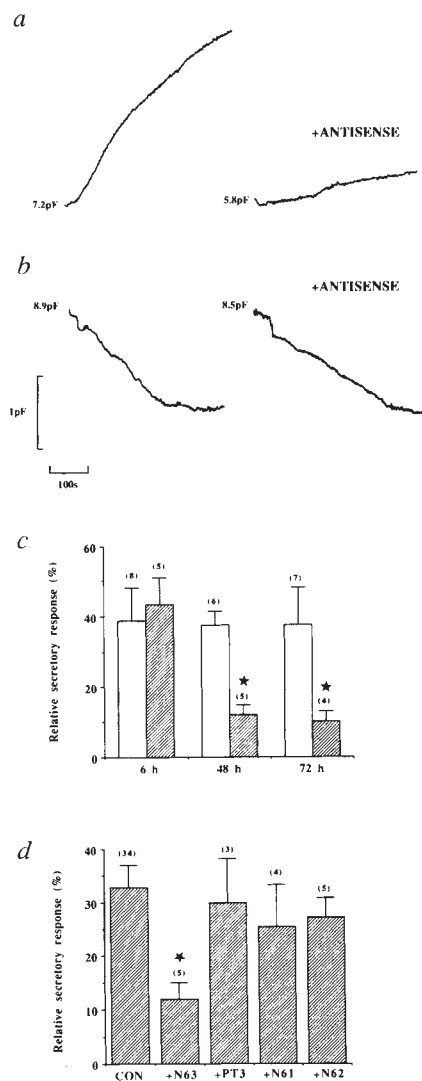


appearance of Rab3-like immunoreactivity. Although cells loaded with N63 do not show any change in immunostaining after 5 h, there is a dramatic reduction in immunoreactivity at 72 h (Fig. 2*b'*). Five days after loading, expression of Rab3-like immunoreactivity had recovered, indicating that antisense suppression of gene expression can be overcome (Fig. 2*c'*). These data are in agreement with measurements of Rab3A turnover in chromaffin cells, in which a lifetime of 54 h was found for a soluble form and of 110 h for soluble and membrane-bound forms (F. Darchen, personal communication). As non-loaded cells or cells loaded with sense (N62) or other antisense probes (PT3 and N61) gave no significant alteration of calcium-triggered exocytosis (Fig. 3*d*), we concluded that N63 inhibits Rab3B synthesis specifically.

Various isoforms of Rab3 proteins have been characterized in different types of secretory cells that exhibit regulated exocytosis^{6–8,25}. These results are consistent with the proposal that the Rab3 subfamily could have a general role in this function, namely regulation of the recruitment and fusion of secretory organelles with the plasma membrane^{26,27}. We have presented converging evidence for the specific involvement of Rab3B in calcium-induced exocytosis in anterior pituitary cells, and it is likely that Rab3 proteins regulate exocytosis downstream of a calcium-dependent event. In support of this, Rab3A effector domain peptides activate exocytotic fusion of granules from non-excitable mast cells¹⁰ and from excitable cells in the absence of cytosolic calcium^{28,29}, indicating that Rab3 activation itself is a calcium-independent rate-limiting process. Therefore

FIG. 3 Representative membrane capacitance (C_m) changes in patch-clamped anterior pituitary cells recorded in control conditions and after loading with DNA oligonucleotides complementary to Rab3A or Rab3B mRNA. C_m measured with the patch clamp technique³¹ is proportional to cell membrane surface area, fluctuations in which are due to the combined processes of exocytosis and endocytosis. *a*, Cell cytosol was dialysed with a solution containing $\sim 1 \mu\text{M}$ Ca^{2+} . Note the reduction in secretory response 48 h after loading with N63 probe (+ ANTISENSE). *b*, Cell cytosol was dialysed with a solution containing about 10^{-8} M Ca^{2+} . The endocytotic response recorded 48 h after loading with N63 (+ ANTISENSE) is not significantly different from the control record. Numbers adjacent to traces in *a* and *b* represent resting C_m . *c*, Effect of N63 probe on calcium-induced secretory response of single pituitary cells recorded after 6, 48 and 72 h after loading (hatched columns; asterisk indicates $P < 0.02$ by Fisher's test). Pipette filling solution as described for *a*. Open columns are control secretory response recorded from cells in the same Petri dish as the loaded cells. *d*, Effect of oligonucleotides N63, PT3, N61 and N62 on calcium-induced secretory response of single anterior pituitary cells. Pipette solution as in *a*. 'CON' denotes the relative secretory response measured in non-loaded cells. Numbers over columns in *c* and *d* represent the number of cells tested. Bars represent standard error of the mean.

METHODS. Oligonucleotide probe loading: Patch-clamp whole-cell recording²² was used to dialyse the cytoplasm with a pipette solution (see below) containing DNA probes¹⁵ (2.7×10^{-8} pM). The injection procedure involved establishing the whole-cell configuration for ~ 2 min, during which time the resting C_m and steady-state current at -70 to -80 mV were recorded. The length of this first recording was chosen to allow equilibration of probe concentration between the pipette medium and the cytosol while the time constant for equilibration of DNA probes was determined³³. This procedure led to the injection of about 50,000 molecules of DNA molecules per cell. Changes in access conductance were continuously monitored during this loading procedure. The patch pipette was removed and the cell membrane resealed. The location of the loaded cell was marked (Fig. 2), and the Petri dish washed with culture medium before incubation for 6–72 h to allow interaction of the injected probe with the cytoplasmic translation machinery. All materials used were sterilized, the medium was filtered and antibiotics were included in the medium. After incubation, cells were used in the second electrophysiological experiment. Out of 131 injected cells, about half were lost during washing and culture, but in 32 cells the secretory response could be determined. During the second recording, a chloride-rich medium was used in the pipette, because the calcium-induced C_m response is enhanced by about twice when compared with data obtained in the presence of a low-chloride pipette medium²⁰. Resting C_m was usually lower than during the second recording (20 of 32 cells), with a nonsignificant average change of resting C_m (-18 ± 15.9 fF; mean $\pm$ s.e.). A small reduction in C_m was expected as at the end of the first recording a part of the plasma membrane was removed with the pipette. Nevertheless, morphological differences were not apparent between cells loaded with probe and non-loaded cells. Steady-state membrane conductance (determined from the steady-state current and driving potential) was slightly smaller in the second recording than in the first. In 10 cells, the average difference between the input conductances was -38 ± 36 pS, not significantly different from zero ($P > 0.05$ with Fisher's test). For comparison, resting conductance during the first electrophysiological examination was 103 ± 26 pS ($n = 10$). Electrophysiology: Cells were voltage-clamped at a holding potential of around -70 mV. C_m was recorded using a dual phase lock-in amplifier (1,600 Hz, 1 mV peak-to-peak) incorporated into a SWAM (Ljubljana, Slovenia) patch-clamp amplifier³⁴. The plots of the passive membrane cell parameters, access conductance, parallel combination of leak and membrane conductance and C_m were derived by a computer-aided reconstruction using an analogue-to-digital converter (CED 1401, Cambridge, UK) to record the signals in digital format on the computer hard



disk (IBM PC compatible). The dc current (low pass, 1–10 Hz, -3 dB), holding potential and the real and imaginary admittance signals (low pass, 1 Hz, -3 dB) were used in the calculation. The software was written by J. Dempster and used the reversal potential for calculating passive parameters³⁵. Recordings were made at room temperature with pipette resistances ranging between 3 to 5 M Ω (measured in KCl-rich solution), giving access conductances of more than 150 nS. The pipette solution contained (in mM): KCl (150); MgCl₂ (2); HEPES (10); EGTA (10.25); CaCl₂ (9); cAMP (0.1); Na₂ATP (2); GTP (0.4); pH to 7.25 with KOH. The calcium activity of this solution was calculated assuming an apparent dissociation constant for the Ca–EGTA complex of 0.15 μM ³⁶ and was found to be close to 1 μM . Where the effect of low-calcium-containing pipette solution was studied, the concentration of CaCl₂ was 1 mM added with 10 mM EGTA, giving a calcium activity around 10^{-8} M. The recording medium contained (in mM): NaCl (127); KCl (5); MgCl₂ (2); CaCl₂ (5); NaH₂PO₄ (0.5); NaHCO₃ (5); HEPES (10); D-glucose (10); pH to 7.25 with NaOH.

small G proteins of the Rab3 subfamily seem to catalyse the molecular processes that specify the interaction between exocytotic organelles and the plasma membrane. □

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Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain

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ONE of the members of the bZIP family of transcriptional activators^{1–5} is NF-IL6/LAP (IL-6 DBP, C/EBP β , CRP2). NF-IL6/LAP protein is highly expressed in liver nuclei², where it has been implicated as a master regulator of the acute-phase response^{1,3,6,7}, induced by interleukin-6 (IL-6) and other inflammatory mediators^{3,8}. Also, NF-IL6/LAP is involved in the activation of the IL-6 promoter in response to IL-1 and bacterial lipopolysaccharide^{1,6}. The control of NF-IL6/LAP expression and activity is complex and poorly understood. Under some conditions the NF-IL6/LAP gene is transcriptionally activated by IL-1 and lipopolysaccharide¹, whereas in other instances, its binding to cognate DNA sequences is enhanced by cytokines^{1,3}. Additionally, the ability of constitutively expressed NF-IL6/LAP to activate transcription is strongly augmented by IL-6, through an unknown signalling pathway. We now show that stimulation of the protein kinase C pathway increases the phosphorylation of Ser 105 within the activation domain of NF-IL6/LAP, and enhances its transcriptional efficacy.

NF-IL6/LAP appears to be involved in induction of several cytokine genes, including IL-6, IL-8, tumour necrosis factor (TNF α) and G-CSF, because critical *cis*-acting elements of these genes include NF-IL6/LAP recognition sequences^{1,9–11}. The signal transduction pathways that mediate activation of these genes

in response to lipopolysaccharide (LPS) and cytokines are not well understood^{1,12,13}, but many of their activities are mimicked by phorbol esters^{10,14,15} which activate protein kinase C (PKC)⁶. Therefore, we used 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and PKC to investigate the post-translational control of NF-IL6/LAP activity. To increase the sensitivity of our system, we cotransfected a CMV-LAP expression vector² encoding NF-IL6/LAP into HepG2 hepatoma cells that express negligible amounts of the endogenous protein, in the presence of a PKC α expression vector¹⁷. Activation of PKC α by TPA led to a small but reproducible increase in total NF-IL6/LAP phosphorylation (Fig. 1a, compare lanes 3 and 4) but had no effect on its expression level (Fig. 1b). The immunoblot shows no production of the alternatively translated LIP protein¹⁸, in the presence or absence of PKC α . Two-dimensional tryptic phosphopeptide analysis¹⁹ of immunopurified NF-IL6/LAP, demonstrated that activation of PKC α by TPA stimulated site-specific phosphorylation (Fig. 1c). To confirm that both endogenous and transiently expressed NF-IL6/LAP are subject to similar changes in their phosphorylation state in a different cell type, we repeated these experiments in a rat fibroblast cell line stably expressing PKC γ that was found to express endogenous NF-IL6/LAP (unpublished data). As observed in HepG2 cells, TPA stimulated site-specific phosphorylation of both endogenous and transiently expressed NF-IL6/LAP (unpublished results). Although the level of several phosphopeptides was increased after TPA treatment, the only change common to both cell types and affecting both endogenous and transiently expressed NF-IL6/LAP was a higher level of phosphopeptide I (Fig. 1c and data not shown).

The migration position of phosphopeptide I appeared identical to that of a phosphopeptide which contains Ser 105 as its phosphoacceptor (our unpublished results). To determine whether Ser 105 is indeed a TPA-responsive phosphorylation site, we substituted its codon with an alanine codon. Vectors expressing wild-type (wt) NF-IL6/LAP or NF-IL6/LAP(Ala105) were transfected into HepG2 cells and the resultant proteins were isolated after *in vivo* labelling with [³²P]orthophosphate. As shown in Fig. 2, substitution of Ser 105 by Ala prevented the appearance of phosphopeptide I.

After identification of Ser 105 as the major TPA-responsive phosphorylation site, we investigated the effect of its phosphorylation on NF-IL6/LAP activity, by comparing the ability of wt NF-IL6/LAP to that of the Ala 105 mutant to activate a NF-IL6/LAP-responsive reporter gene (Fig. 3). Although both proteins were expressed (Fig. 3c) and translocated to the nucleus at very similar levels (data not shown), the wt protein was a more efficient activator of a CAT-reporter linked to a NF-IL6/LAP recognition sequence^{1,2,20} than the Ala 105 mutant (Fig. 3a). Cotransfection of the wt NF-IL6/LAP expression vector with a PKC α expression vector, and TPA treatment resulted in a five-fold increase in transactivation, while transactivation by NF-IL6/LAP(Ala105) was not affected (Fig. 3b). The effect of Ser 105 phosphorylation on transactivation can be mimicked by introduction of a negatively charged residue; a mutant NF-IL6/LAP(Asp 105) is a several-fold more potent activator than the wt protein (Fig. 3a). To assess whether increased activity of NF-IL6/LAP following phosphorylation of Ser 105 is due to enhanced DNA-binding affinity, we did mobility shift assays. Increasing amounts of nuclear extracts of cells transfected with the various NF-IL6/LAP vectors were incubated with a ³²P-labelled oligonucleotide spanning the NF-IL6/LAP recognition sequence. Specific binding was greatly increased by transient transfection of both wt and mutant NF-IL6/LAP expression vectors (Fig. 3c). TPA-treatment and the substitution of Ser 105 by Ala and Asp had no effect on DNA-binding activity (Fig. 3d). (Specificity of binding was demonstrated by competition and antibody super-shift experiments; data not shown.) These results strongly suggest that phosphorylation of Ser 105 affects the activation function of NF-IL6/LAP.

To confirm that phosphorylation of Ser 105 potentiates the

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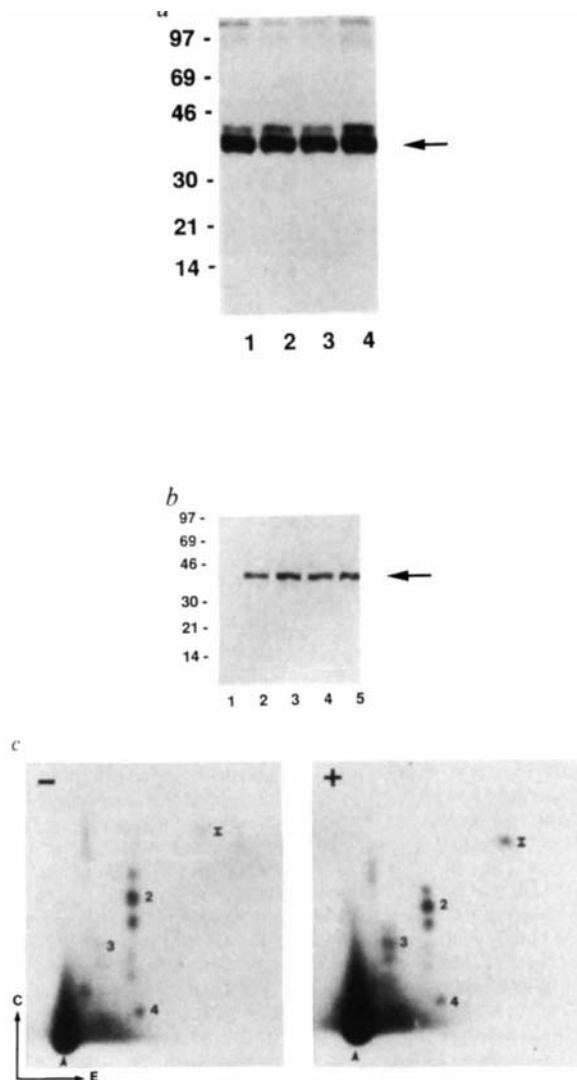


FIG. 1 Activation of the PKG pathway induces site-specific phosphorylation of NF-IL6/LAP. *a*, HepG2 cells were transiently transfected with a CMV-LAP expression vector, incubated with [32 P]orthophosphate, and 32 P-labelled NF-IL6/LAP was purified by immunoprecipitation using anti-LAP antibodies. The cells were either cotransfected with pUC19 (lane 1), pCDM8-0 (lane 2; empty expression vector) or pCDM8-PKC α (lanes 3 and 4). Cells used in lane 4 were stimulated with TPA (100 ng ml $^{-1}$) for 20 min before collecting. *b*, In a parallel experiment, HepG2 cells were either mock transfected (lane 1) or transfected with the CMV-LAP expression vector (lanes 2–5). The cells were cotransfected with pUC19 (lane 2), pCDM8-0 (lane 3) or pCDM8-PKC α (lanes 4 and 5). Cells used in lane 5 were treated for 20 min with TPA (100 ng ml $^{-1}$) before collecting. Nuclear extracts were prepared and 10 μ g samples were analysed by western blotting using anti-LAP antibodies. *c*, Tryptic phosphopeptide maps of *in vivo* labelled NF-IL6/LAP. Equal amounts of NF-IL6/LAP were isolated from HepG2 cells transfected as described in *a* (lanes 3 and 4) that were either untreated (–) or treated (+) with TPA, and digested with trypsin. The resultant peptides were separated by high-voltage electrophoresis (horizontal dimension) followed by ascending thin layer chromatography (vertical dimension), and visualized by autoradiography. The levels of both phosphopeptides 1 and 3 are increased after PKC activation. But only phosphopeptide 1 is reproducibly increased in response to TPA-treatment of another cell type (data not shown). Only the reproducibly observed NF-IL6/LAP-derived phosphopeptides are numbered; other phosphopeptides are probably derived from contaminating proteins. The arrowhead indicates the origin.

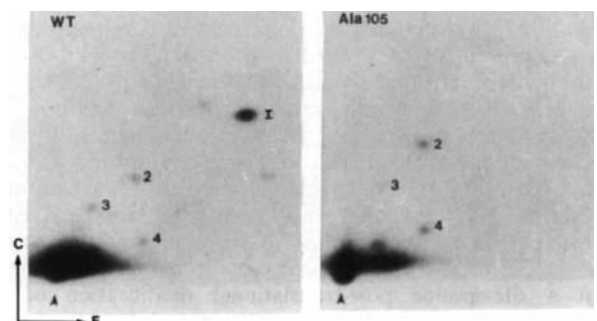
METHODS. Subconfluent cultures of HepG2 cells were transfected using the calcium-phosphate method as described^{2,20}, with pCMV-LAP(wt) (1.0 μ g), pCDM8-0 (3.0 μ g) and pCDM8-PKC α (3.0 μ g)¹⁷. After transfection, cells were kept in medium alone for 40 h and labelled with [32 P]-orthophosphate (2.5 mCi ml $^{-1}$) for 4 h. TPA (100 ng ml $^{-1}$) was added as indicated for the last 20 min. Cells were lysed in RIPA buffer, NF-IL6/LAP was immunoprecipitated with a specific antibody² and separated by SDS-polyacrylamide-gel-electrophoresis as described²⁵. After blotting onto nitrocellulose, *in vivo* labelled LAP was digested with trypsin. The digested peptides were eluted from the membrane, spotted on a TLC plate and separated by two-dimensional electrophoresis as described¹⁹. Nuclear extracts were prepared from HepG2 cells transfected as for 32 P-labelling using described procedures², and analysed by western blotting using anti-LAP antibodies². The antigen-antibody complexes were visualized using the ECL detection system as recommended by the manufacturer (Amersham). CMV-LAP encodes for the form of NF-IL6/LAP which is mainly translated in the liver starting at the second ATG of the NF-IL6/LAP open reading frame¹⁸.

activation function of NF-IL6/LAP, we fused both the wt and mutant versions (Ala 105 and Asp 105) of its N-terminal activation domain¹⁸ to the GAL4 DNA-binding domain²¹ (Fig. 4a). The ability of LAP (Ser 105)/GAL4 to activate the GAL4-responsive reporter (5 $\times$ GAL4-LUC) was very similar to that of LAP(Ala 105)/GAL4, and both were 50- to 200-fold more active than the GAL4 DNA-binding domain alone (Fig. 4b). By contrast, LAP(Asp 105)/GAL4, was three-fold more active than LAP(Ser 105)/GAL4. The activity of LAP(Ser 105)/GAL4 was stimulated fourfold by activation of PKC α , whereas the activity of LAP(Ala 105)/GAL4 was only slightly increased (Fig. 4c).

These results demonstrate that the activation potential of NF-IL6/LAP is directly enhanced by phosphorylation of Ser 105, an effect mimicked by introduction of a negative charge into that position.

The protein kinase responsible for phosphorylation of Ser 105 is unlikely to be PKC itself, because this residue is not phosphorylated by purified PKC *in vitro* (our unpublished results). In addition, a constitutively activated derivative of PKC α that is predominantly nuclear¹⁷ does not stimulate NF-IL6/LAP activity or phosphorylation (unpublished results). This derivative, however, is capable of affecting the phosphorylation and

FIG. 2 The PKC-stimulated phosphorylation site of NF-IL6/LAP is Ser 105. HepG2 cells were transiently transfected with pCMV-LAP(wt) or pCMV-LAP (Ala 105), and labelled with [32 P]orthophosphate (2.5 mCi ml $^{-1}$) for 4 h. Following treatment with TPA (100 ng ml $^{-1}$), as described in Fig. 1, cells were lysed in RIPA buffer. Wild-type and mutant NF-IL6/LAP were isolated from equal amounts of cell lysates by immunoprecipitation with anti-LAP antibodies². After tryptic digest, peptides were separated by two-dimensional electrophoresis as described above.



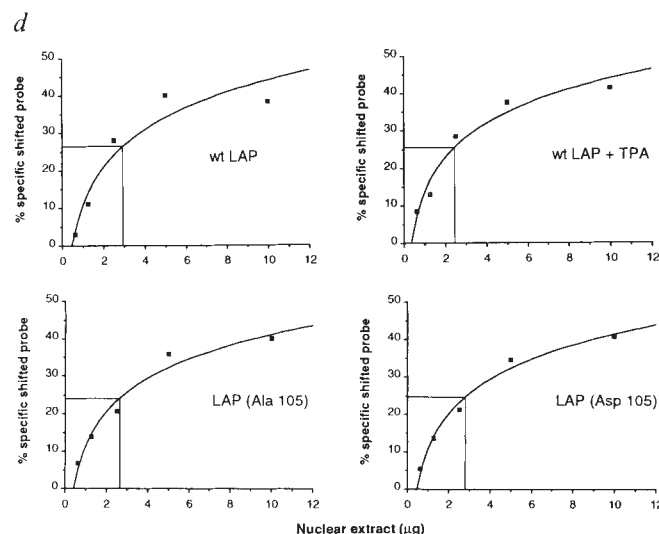
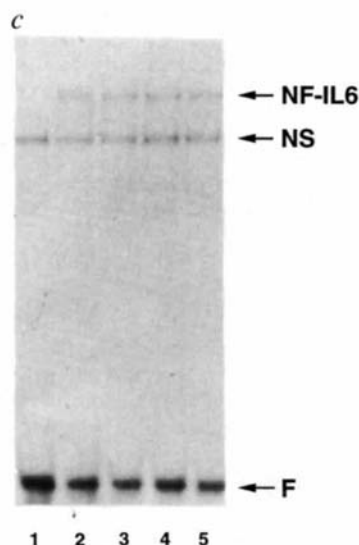
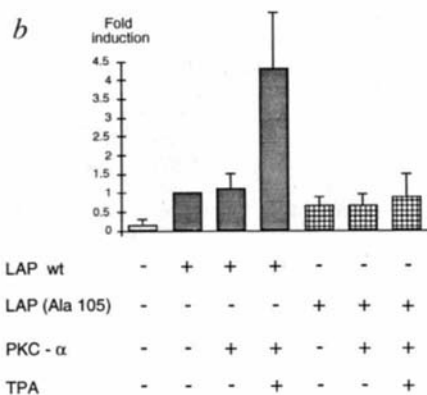
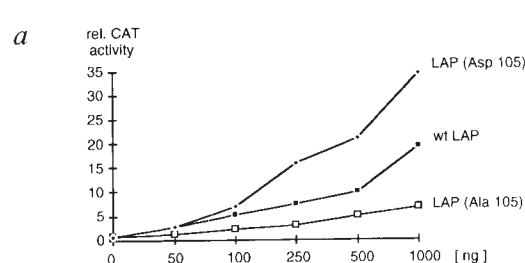


FIG. 3 Phosphorylation of NF-IL6/LAP on Ser 105 enhances its activation function. *a*, The NF-IL6/LAP-responsive D-CAT-reporter plasmid^{2,20} (3 μ g) was cotransfected into HepG2 cells with increasing amounts of pCMV-LAP(wt), pCMV-LAP (Ala 105) and pCMV-LAP (Asp 105) expression vectors as indicated. Forty-eight hours later the cells were collected and CAT activity was determined. The results shown are the averages of three experiments. *b*, HepG2 cells were cotransfected with 3 μ g of the D-CAT reporter and pCMV-LAP(wt), pCMV-LAP (Ala 105), pCDM8-0 and pCDM8-PKC α expression vectors (1 μ g each), as indicated. Serum-starved cells were stimulated with TPA (100 ng ml⁻¹), as indicated, 20 h after transfection. Cells were collected 40 h after transfection and CAT expression was measured. The results shown are the averages of three experiments. *c*, HepG2 cells were co-transfected with 1 μ g of each of pCDM8-0 (lane 1), pCMV-LAP(wt)+pCDM8-PKC α (lanes 2 and 3), pCMV-LAP(wt)+pCDM8-0 (lane 4), pCMV-LAP(Asp105)+pCDM8-0 (lane 5). Nuclear extracts of the cells were prepared 20 h after transfection. The cells in lane 3 were stimulated with TPA (100 ng ml⁻¹) 20 min before collecting. Mobility shift assays were done using 15 pg of a ³²P-labelled oligonucleotide spanning the D-site (oligo D) of the albumin promoter. The migration positions of the NF-IL6/LAP and a nonspecific (NS) protein-DNA complex are indicated, as well as the free (F) probe. The figure shows a representative gel shift assay where ~10% of the probe was specifically shifted by NF-IL6/LAP. *d*, A fixed amount (15 pg) of the ³²P-labelled oligo D was incubated with increasing amounts of nuclear extracts (in μ g) under the same conditions shown above. The fraction of the specifically bound probe was quantified by an Ambis gel scanner and plotted as a function of amount of nuclear extract. The amount of nuclear extracts required to give 50% occupancy is indicated. METHODS. After washing the cells twice with ice-cold phosphate-buffer saline, nuclear extracts were prepared with minor modifications according to ref. 31. Nuclear extracts were incubated with a ³²P-labelled oligonucleotide spanning the D-site of the albumin promoter as described earlier². Free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel.

activity of other nuclear proteins such as myogenin²². In addition, although Ser 105 is phosphorylated by PKA *in vitro*, elevation of intracellular cAMP does not stimulate Ser 105 phosphorylation (C.T. and P.V., unpublished results). Most probably, activation of PKC results in activation of downstream protein kinases including the one directly responsible for phosphorylation of NF-IL6/LAP on Ser 105. Here, PKC activation only serves to mimic part of the signalling response which may be elicited by inflammatory mediators.

These studies provide insights into the mechanisms by which the activity of NF-IL6/LAP could be potentiated by IL-6, IL-1 and LPS to affect the activation of cytokine^{1,6,9-11} and acute-phase response^{3,7} genes. It was shown that transcription from reporter genes bearing NF-IL6/LAP recognition sequences does not require *de novo* protein synthesis^{1,3}. We now demonstrate that a site-specific post-translational modification of NF-IL6/LAP enhances its ability to activate such target genes.

The PKC pathway is activated by inflammatory mediators^{15,16}, including TNF α ²³. Because this pathway does not affect DNA-binding by NF-IL6/LAP, the enhancement of DNA-binding observed previously^{1,6,7} is likely to be mediated by a different signalling pathway. In this respect, NF-IL6/LAP resembles the c-Jun protein whose DNA-binding and transcriptional enhancement activities are regulated by separate signalling pathways^{24,25}. In addition to c-Jun^{25,26} and CREB²⁷, NF-IL6/LAP is the third eukaryotic transcriptional regulator whose ability to activate transcription is positively regulated by phosphorylation, further proving the generality of this regulatory mechanism²⁸.

Recently NF-IL6/LAP was found to bind to the serum response element of the *c-fos* promoter and contribute to its induction by cAMP²⁹. Although this response may be unique to pheochromocytoma cells²⁹, the ability of NF-IL6/LAP to respond to several different signalling pathways, including the

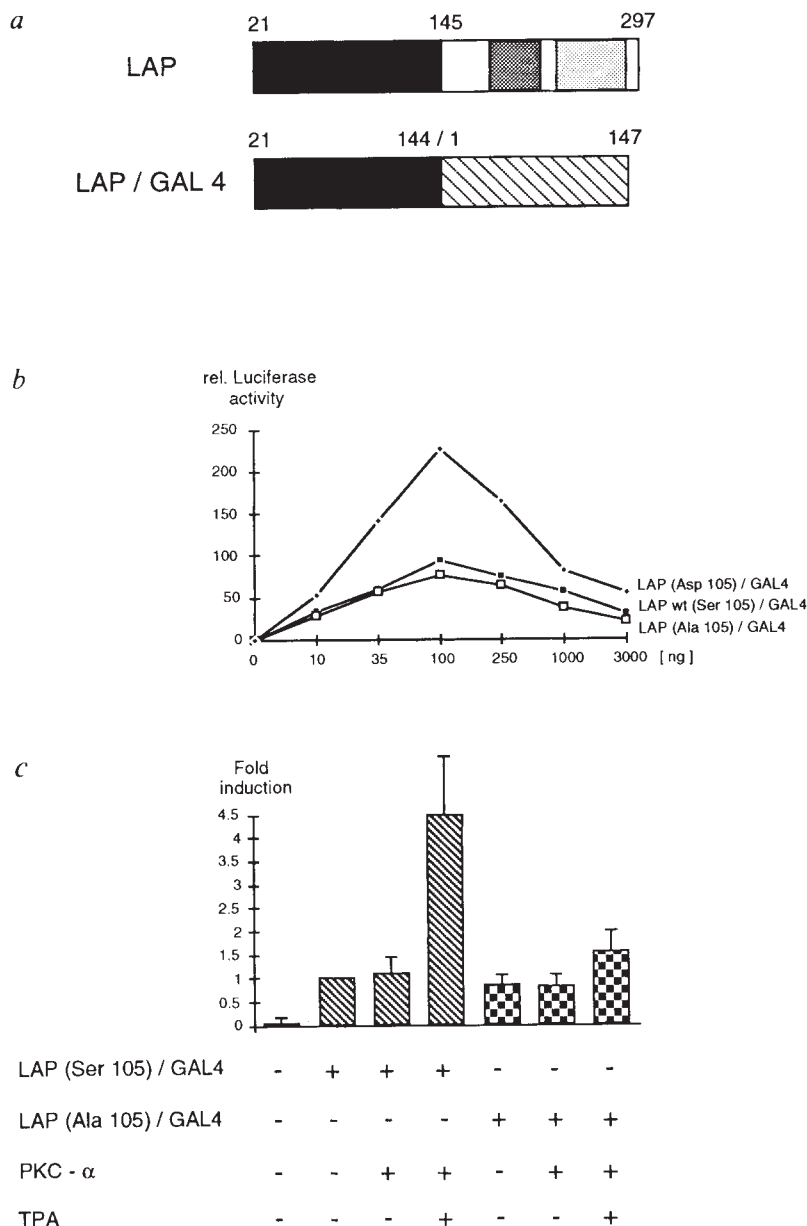


FIG. 4 Phosphorylation of Ser 105 augments the activity of the NF-IL6/LAP activation domain. **a**, The transactivation domain of NF-IL6/LAP (amino acids 21–144) (solid bar), was ligated to the N terminus of the yeast GAL4 DNA-binding domain (amino acids 1–147) (hatched bar) to generate the chimaeric activator LAP/GAL4. **b**, The GAL4-responsive reporter 5 × GAL4-LUC (3 µg) was cotransfected with increasing amounts of pSV40-LAP (Ser 105)/GAL4, pSV40-LAP (Ala 105)/GAL4, or pSV40-LAP(Asp 105)/GAL4 expression vectors. After 40 h the cells were collected and luciferase activity was determined. The maximal luciferase activity produced by the 5 × GAL4-LUC reporter cotransfected with pSV40-LAP(Ser 105)/GAL4 was considered to be 100%. The results shown are averages of two experiments. **c**, The 5 × GAL4-LUC reporter (3 µg) was cotransfected with pSV40-LAP(Ser 105)/GAL4 (10 ng), pSV40-LAP (Ala 105)/GAL4 (10 ng), pCDM8-PKCα (100 ng) as indicated. After transfection, the cells were serum-starved for 20 h and then were treated with TPA (100 ng ml⁻¹) for 20 h or left untreated. Cell extracts were prepared and luciferase activity was determined. The results represent the averages of three different experiments.

METHODS. To construct the GAL4 fusion proteins, the NcoI–EcoRI fragments from pET8c-LAP (Ser 105) and pET8c-LAP (Ala 105) (ref. 2), were replaced by the BspHI–EcoRI fragment containing the coding sequence of the GAL4 DNA-binding domain (amino acids 1–147) from a modified version of the pSG424 vector²¹, generated by T. Deng (personal communication). The chimaeric LAP-GAL4 open reading frames were excised by BglII–EcoRI digestion and were cloned into the pSG424 vector from which the GAL4 DNA-binding domain was removed.

one involving PKC, may contribute to the induction of *c-fos* by many different stimuli. Collectively, our results indicate that phosphorylation of NF-IL6/LAP at Ser 105 following activation of the PKC pathway, may be critical for transactivation of cytokine and acute-phase reactant genes in response to inflammation and infection^{1,3,8,30}. □

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CAP interacts with RNA polymerase in solution in the absence of promoter DNA

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PROTEIN-PROTEIN interactions between transcription activator proteins and RNA polymerase or basal transcription factors have been suggested to be important for transcription activation¹⁻⁸. Interactions between catabolite gene activator protein (CAP)^{9,10} and RNA polymerase have been proposed based on face-of-helix-dependent transcription activation by CAP¹¹⁻¹³ and based on face-of-helix-dependent cooperative binding of CAP and RNA polymerase to promoter DNA^{14,15}. Mutants of CAP specifically defective in transcription activation have been isolated (mutants defective in transcription activation, but not defective in DNA binding and DNA bending¹⁶⁻¹⁹). All such mutants contain amino-acid substitutions within a surface loop consisting of amino acids 152 to 166 of CAP¹⁶⁻¹⁹. Here we use the thermodynamically rigorous technique of fluorescence polarization²⁰⁻²³ to show that CAP interacts with RNA polymerase in solution in the absence of promoter DNA ($K_{D,app} = 2.8 \times 10^{-7}$ M), whereas [Ala158]CAP, a mutant of CAP specifically defective in transcription activation, does not.

We have used the following components: (1) CAP, (2) a fluorochrome-labelled 42-base-pair oligodeoxyribonucleotide duplex containing the consensus DNA site for CAP²⁴ and containing no known specific determinants for binding of RNA polymerase ('ICAP42FL'; Fig. 1), and (3) *Escherichia coli* RNA polymerase holoenzyme ('RNAP'). We have measured fluorescence anisotropy of the CAP-ICAP42FL complex in the absence of RNAP and in the presence of 75 nM to 1,200 nM RNAP. Our experimental design was premised on the large difference in size, and thus the large difference in rotation rates and in fluorescence anisotropies, of a CAP-ICAP42FL binary complex ($M_r \sim 73$ K) versus a CAP-ICAP42FL-RNAP ternary complex ($M_r \sim 520$ K).

Results of fluorescence polarization experiments with wild-type CAP are presented in Fig. 2a. Addition of RNAP to the preformed wild-type CAP-ICAP42FL complex results in a large (40%), saturable increase in fluorescence anisotropy. In contrast, addition of RNAP to ICAP42FL alone does not result in a large increase in fluorescence anisotropy. We conclude that RNAP interacts with the wild-type CAP-ICAP42FL complex in solution. The dissociation constant for the interaction was calculated by nonlinear regression analysis of the data assuming that the interaction occurs according to the simplest possible scheme: CAP-ICAP42FL + RNAP $\rightleftharpoons$ CAP-ICAP42FL-RNAP. The dissociation constant for the interaction is $2.8 (\pm 0.8) \times 10^{-7}$ M. It is noteworthy that the dissociation constant for the interaction is comparable to the concentration of RNAP *in vivo*²⁵. We conclude that interaction between CAP and RNAP in the absence of promoter DNA would be able to occur *in vivo*.

Results of fluorescence polarization experiments with [Ala158]CAP, a mutant of CAP specifically defective in transcription activation^{18,19}, are presented in Fig. 2b. Addition of RNAP to the preformed [Ala158]CAP-ICAP42FL complex

does not result in a large increase in fluorescence anisotropy. Indeed, the results upon addition of RNAP to the preformed [Ala158]CAP-ICAP42FL complex are not significantly different from the results upon addition of RNAP to ICAP42FL alone. We conclude that RNAP does not detectably interact with the [Ala158]CAP-ICAP42FL complex in solution. This implies that RNAP exhibits a >40-fold higher affinity for the wild-type CAP-ICAP42FL complex than for the [Ala158]CAP-ICAP42FL complex; this corresponds to a >2 kcal mol⁻¹ higher binding free energy. We conclude also that the interaction between RNAP and the wild-type CAP-ICAP42FL complex in solution requires the surface loop consisting of amino acids 152 to 166 of CAP.

Control fluorescence polarization experiments establish that under our standard experimental conditions, both wild-type CAP and [Ala158]CAP fully saturate oligodeoxyribonucleotide duplex ICAP42FL (Fig. 3a) and establish that, within experimental error, wild-type CAP and [Ala158]CAP bind to oligodeoxyribonucleotide duplex ICAP42FL with identical affinities (Fig. 3b; compare ref. 18). Electrophoretic mobility shift DNA-bending experiments¹⁸ and protein-DNA fluorescence energy transfer DNA-bending experiments (unpublished data) indicate that wild-type CAP and [Ala158]CAP bend DNA to the same extent.

Additional control fluorescence polarization experiments establish that in the CAP, ICAP42FL and RNAP concentration ranges in Fig. 2a, formation of the CAP-ICAP42FL-RNAP ternary complex requires cyclic AMP, the allosteric effector required for sequence-specific DNA binding by CAP and transcription activation by CAP⁹ (unpublished data).

Although CAP-RNAP interactions have been previously analysed using fluorescence polarization of a fluorochrome-labelled CAP derivative and RNAP (ref. 26) and other studies also suggested that CAP interacts with RNAP in the absence of promoter DNA (ref. 27), no evidence for the specificity of the interaction was presented. Our results establish a direct correlation between the ability to activate transcription and the ability to interact with RNAP in solution in the absence of promoter DNA and thus constitute strong evidence that transcription acti-

F - p - GCAACGCAATAAATGTGATCTAGATCACATTTTAGGCACCCC
CGTTGCGTTATTTACACTAGATCTAGTGTAAAAATCCGTGGGG - p

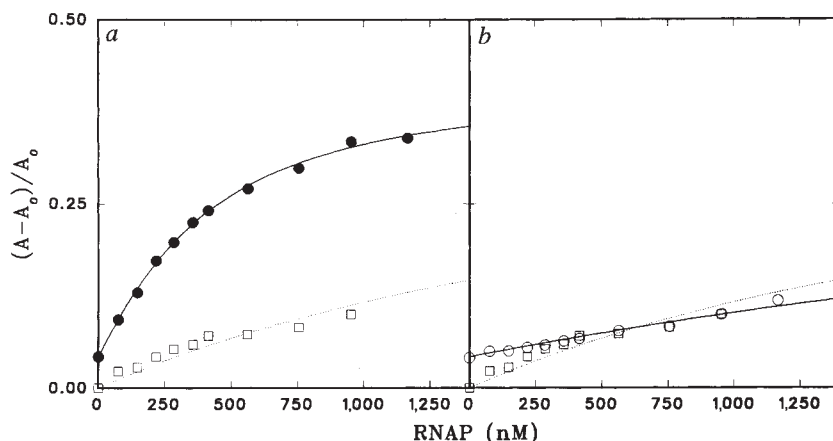
FIG. 1 Fluorochrome-labelled 42-base-pair oligodeoxyribonucleotide duplex containing the consensus DNA site for CAP²⁴ and containing no known specific determinants for binding of RNA polymerase (ICAP42FL). 'F' denotes the reporter group (acetamido-5-fluorescein).

METHODS. A 5'-phosphorothioate oligodeoxyribonucleotide corresponding to the top strand of ICAP42FL and a 5'-phosphate oligodeoxyribonucleotide corresponding to the bottom strand of ICAP42FL were synthesized using solid-phase β -cyanoethylphosphoramidite chemistry on an AB380A automated synthesizer. 5'-Phosphorothioate and 5'-phosphate were introduced using 2-[2-(4,4'-dimethoxytrityloxy)ethylsulphonyl]ethyl-(2-cyanoethyl)-(N,N'-diisopropyl)-phosphoramidite (Glen Research) and oxidation with, respectively, tetraethylthiuram disulphide (Applied Biosystems) or I₂/water/pyridine. Products were purified using denaturing polyacrylamide gel electrophoresis followed by reversed-phase chromatography on Sep-Pak C18 (Waters). A fluorescein-labelled oligodeoxyribonucleotide corresponding to the top strand of ICAP42FL was prepared by reaction of 5-iodoacetamido-fluorescein (Molecular Probes) with the 5'-phosphorothioate oligodeoxyribonucleotide (compare ref. 28). Reaction mixtures contained (260 μ l): 1.4 mM 5-iodoacetamido-fluorescein, 0.033 mM 5'-phosphorothioate oligodeoxyribonucleotide, 10 mM sodium phosphate (pH 7.0), and 9% dimethylformamide. Reactions proceeded for 3 h at 37 °C under N₂. Product was purified using ethanol precipitation followed by reversed-phase HPLC on RP-18 (EM Science). Top-strand and bottom-strand oligodeoxyribonucleotides were annealed as described²⁴.

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FIG. 2 Fluorescence polarization analysis of CAP-ICAP42FL-RNAP interaction. *a*, Data for wild-type CAP. Filled circles, titration of wild-type CAP-ICAP42FL complex with RNAP; squares, control titration of ICAP42FL with RNAP. Solid line represents the best fit of the data to the equation describing formation of the 1:1:1 CAP-ICAP42FL-RNAP ternary complex from CAP-ICAP42FL and RNAP (calculated as described²¹). The dissociation constant for the interaction is $2.8 (\pm 0.8) \times 10^{-7}$ M. *b*, Data for [Ala158]CAP. Circles, titration of [Ala158]CAP-ICAP42FL complex with RNAP; squares, control titration of ICAP42FL with RNAP.

METHODS. CAP and [Ala158]CAP were purified by cAMP affinity chromatography as described²⁹. RNAP was purified from *Escherichia coli* K-12 strain X7029 ($\Delta lacX74$ *thi*) by DNA-cellulose chromatography, Sephacryl S-300 chromatography, and Mono Q FPLC as described³⁰. Reaction mixtures contained (120 μ l): 350 nM CAP or [Ala158]CAP (where indicated), 250 nM ICAP42FL, 0 to 1,200 nM RNAP, 10 mM MOPS-NaOH (pH 7.2), 250 mM NaCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM cAMP and 5% glycerol. Components except RNAP were incubated for 15 min at 25 °C. Reactions were initiated by addition of RNAP and were carried out for 4 min at 25 °C. Fluorescence polariza-



tion measurements were made on a SLM8000C spectrofluorimeter with T optics²¹. Samples were excited at 490 nm and emission was observed using KV 550 filters (Schott, Inc.). Data were expressed as $(A-A_0)/A_0$, where A denotes the fluorescence anisotropy in the presence of the indicated concentration of RNAP, and A_0 denotes the fluorescence anisotropy in the absence of RNAP and CAP.

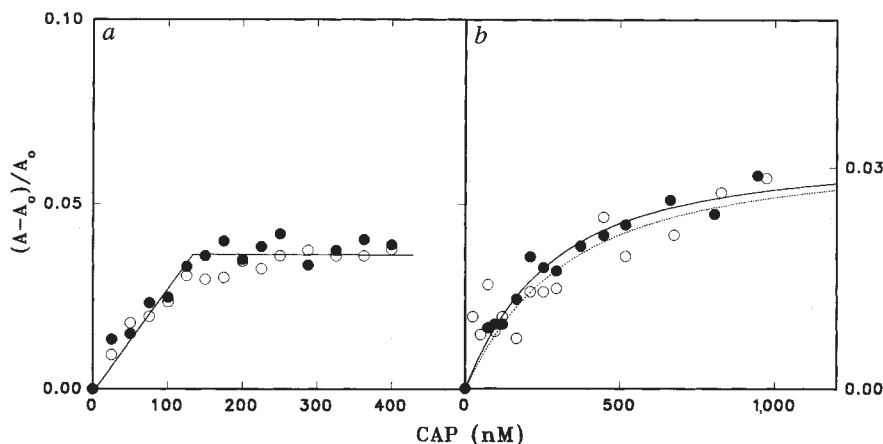


FIG. 3 Fluorescence polarization analysis of CAP-ICAP42FL interaction. Filled circles, titration of ICAP42FL with wild-type CAP; open circles, titration of ICAP42FL with [Ala158]CAP. **METHODS.** The concentration of ICAP42FL was 125 nM. Titrations in *a* were carried out under our standard experimental conditions ([NaCl], 200 mM; Fig. 2). Titrations in *b* were carried out under high-salt experimental conditions ([NaCl], 600 mM). Solid lines and dotted lines represent best fits of the data for, respectively, wild-type CAP and [Ala158]CAP (calculated as described²¹). Binding constants under high-salt experimental conditions are: wild-type CAP, $5 (\pm 2) \times 10^6$ M⁻¹; [Ala158]CAP, $4 (\pm 3) \times 10^6$ M⁻¹.

vation by CAP requires a direct protein-protein interaction with RNA polymerase. As a fluorochrome can be attached to a synthetic oligodeoxynucleotide duplex of any sequence, the

fluorescence polarization method of this report should be broadly generalizable to transcription activator proteins that interact with known specific DNA sites. □

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An RNA motif that binds ATP

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RNAs that contain specific high-affinity binding sites for small molecule ligands immobilized on a solid support are present at a frequency of roughly one in 10^{10} – 10^{11} in pools of random sequence RNA molecules^{1,2}. Here we describe a new *in vitro* selection procedure designed to ensure the isolation of RNAs that bind the ligand of interest in solution as well as on a solid support. We have used this method to isolate a remarkably small RNA motif that binds ATP, a substrate in numerous biological reactions and the universal biological high-energy intermediate. The selected ATP-binding RNAs contain a consensus sequence, embedded in a common secondary structure. The binding properties of ATP analogues and modified RNAs show that the binding interaction is characterized by a large number of close contacts between the ATP and RNA, and by a change in the conformation of the RNA.

In vitro selection experiments involve the construction of a large pool of random polynucleotide sequences, followed by repeated cycles of enrichment for species with the desired properties and amplification of the enriched pool. We started with a pool of RNAs 169 nucleotides long, with a complexity of $\sim 10^{14}$

different sequences². RNA molecules able to bind ATP were selected by affinity chromatography on ATP-agarose columns, with the ATP linked to the agarose at its C8 position (Fig. 1a). RNA molecules that were retained by the ATP-agarose matrix were affinity-eluted with ATP. We expected that this free ATP would compete with the ATP-agarose for occupancy of the ATP-binding site of RNA sequences capable of binding ATP in solution; thus such sequences would be eluted from the column, whereas sequences capable of binding to the ATP-agarose column but incapable of binding free ATP would be retained. The eluted RNA was amplified by reverse transcription and polymerase chain reaction (PCR). *In vitro* transcription of the double-stranded DNA templates obtained in this manner yielded an enriched pool of RNAs for a new cycle of selection. After six rounds of selection and amplification, a population of RNAs that eluted specifically with ATP, but not with GTP, ITP, CTP or UTP, was obtained. Selection was continued for two additional rounds to eliminate any remaining inactive species.

We sequenced 39 clones from the eighth cycle RNA population and found 17 different sequences, of which the most abundant sequence (C8-ATP-3) occurred 14 times, and 12 sequences occurred just once (Fig. 1b). Comparison of the seventeen different sequences revealed an 11-nucleotide consensus sequence, of which seven positions are invariant among all clones but one (C8-ATP-15). Clones 2, 3, 8, 15 and 19 were individually tested for binding to ATP-agarose. All had a dissociation constant (K_d) of less than 50 μ M, except for C8-ATP-15, for which

FIG. 1 a, Selection of ATP-binding RNAs. A chemically synthesized pool of DNA molecules containing a central region of 120 random nucleotides flanked by constant regions used as primer binding sites² was PCR-amplified and transcribed *in vitro* by T7 RNA polymerase in the presence of [α -³²P]GTP. RNA was ethanol-precipitated and unincorporated nucleotides removed by Sephadex-G50 chromatography. Following a brief incubation at 65 °C in binding buffer (300 mM NaCl, 20 mM Tris, pH 7.6, 5 mM MgCl₂), the RNA was cooled to room temperature before being loaded onto a 1-ml ATP-agarose affinity column. The affinity matrix contained ~ 1.6 mM ATP linked through its C8 position through a diamino-hexyl linker to cyanogen bromide-activated agarose (Sigma). After washing with 6 column-volumes of binding buffer, bound RNAs were eluted with 3 column-volumes of binding buffer containing 4 mM ATP, then concentrated by precipitation with ethanol. For the first three cycles, an agarose pre-column was used to prevent enrichment of the RNA pool with agarose-binding RNAs, and bound RNAs were eluted with 5 mM EDTA in water rather than affinity-eluted with ATP. After reverse transcription and PCR amplification, DNA templates were transcribed and the resulting RNA was used in the next round of selection. b, Sequences of ATP-binding RNAs. RNA from the eighth round of selection was converted to cDNA, amplified as double-stranded DNA by PCR, digested with *Eco*RI and *Bam*HI, gel-purified and cloned into the phage M13 based vector pGem3Z (Promega). Individual clones were sequenced by the dideoxy method. Sequences were aligned relative to the consensus sequence. The 11-base consensus motif (boxed) and the conserved G are in large bold type, flanked by nucleotides (underlined) that form two base-paired stems. Clone identification numbers are given to the left of each sequence, and the number of clones with each sequence are shown in parenthesis on the right.

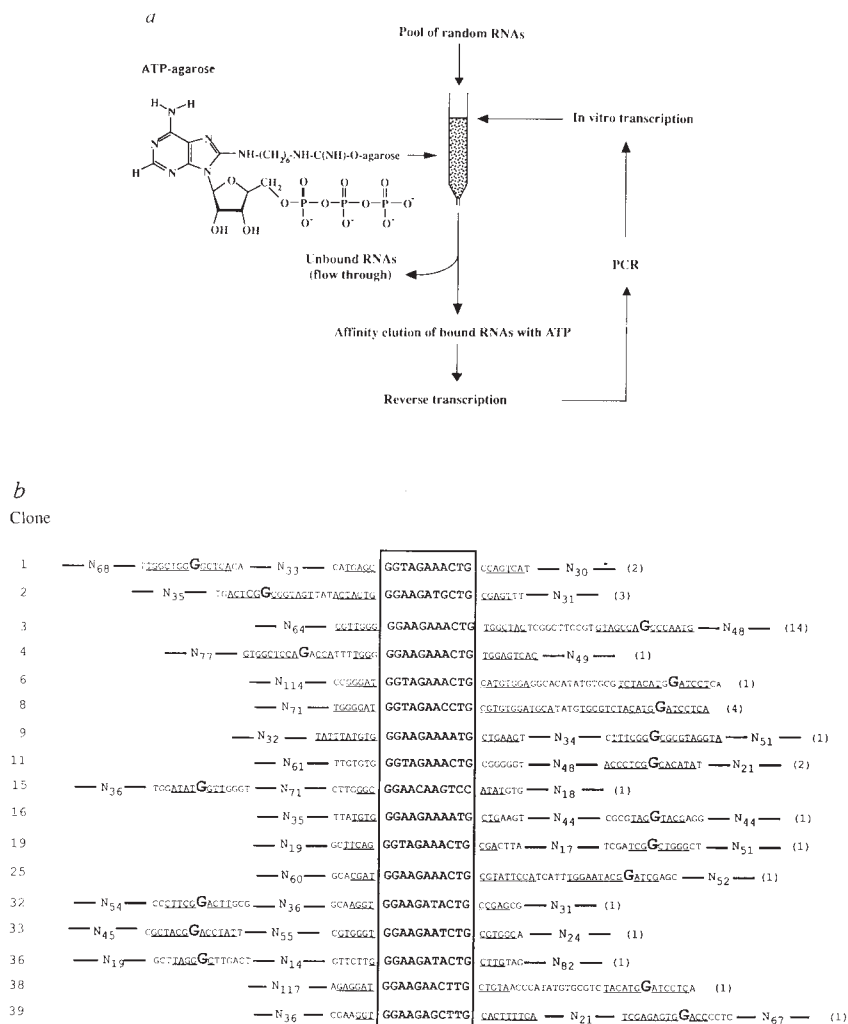
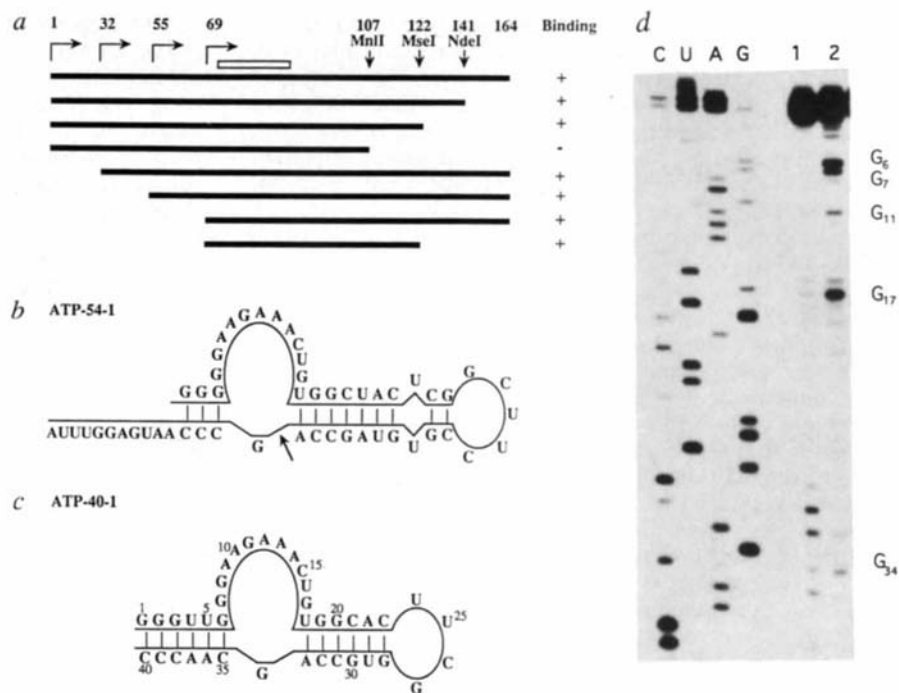


FIG. 2 Secondary structure of the ATP-binding domain. *a*, Deletion analysis of clone C8-ATP-3. 3' deletions were generated by restriction digestion of the template double-stranded DNA with the indicated restriction enzymes before transcription. 5' deletions were generated by nested PCR. The position of the consensus sequence is indicated by the open box. Transcripts were purified on a denaturing 10% acrylamide gel before being tested for binding. *b*, Secondary structure of ATP-54-1, the smallest active RNA generated by deletion. Arrow indicates the site of cleavage by *Mnl*I. *c*, Secondary structure of ATP-40-1, the 40-nucleotide version of the ATP-binding domain. *d*, Kethoxal modification. A 57-base long RNA carrying an additional 17 bases (-UAUG-UGCGGAUCCUCA-3') at the 3' end of ATP-40-1 was used to map sites accessible to kethoxal. Kethoxal (40 μ l, 33 mg ml⁻¹ in 30% ethanol) was added to 4 μ g RNA in 80 μ l buffer (80 mM sodium cacodylate, pH 7, 300 mM NaCl, 20 mM magnesium acetate) containing either 1 mM dATP or 1 mM ATP. After 10 min incubation at room temperature, 0.1 vol ice-cold 0.5 M potassium borate, pH 8.1, was added to stop the reaction and the RNA was purified by ethanol precipitation. The sites of modification by kethoxal were mapped by primer extension at 48 °C with reverse transcriptase (Life Sciences) using a ³²P-labelled 17-mer synthetic primer complementary to the 3' end of the 57-mer RNA. The



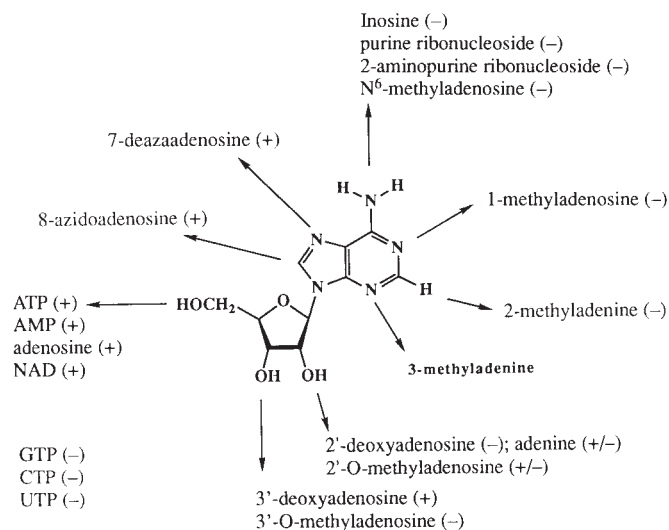
RNA was hydrolysed in NaOH and the DNA products analysed on a denaturing 12% polyacrylamide gel. Lanes C, U, A, G refer to the sequencing lanes obtained by reverse transcription of the RNA at 48 °C in the presence of dideoxy NTPs. Lanes 1 and 2 show the pattern of kethoxal modification in the presence of 1 mM dATP and 1 mM ATP respectively.

the estimated K_d was ~250 μ M (ref. 3).

To determine the minimal sequence for ATP binding, deletions of C8-ATP-3 were analysed (Fig. 2*a*). An active RNA molecule 54 nucleotides in length (ATP-54-1) was generated by a combination of 5' and 3' deletions. This RNA can be folded into a secondary structure in which the 11-base consensus is flanked by two base-paired stems (Fig. 2*b*). Deletion of the left-hand stem abolished ATP-binding activity; dimethylsulphate

modification experiments also supported the proposed secondary structure. Comparing sequences of all the clones showed that they all had a potential to fold into this secondary structure (Fig. 1*b*). This analysis also highlighted the presence of an invariant unpaired G opposite the 11-base consensus. The orientation and distance of this G and its flanking sequences relative to the consensus sequence was variable from clone to clone. The stems flanking the conserved G and the consensus were variable in

FIG. 3 Ligand specificity. Positions involved in interaction with the RNA were determined by the ability of ATP analogues to elute bound RNA from an ATP-agarose column. 1-ml fractions were collected following the loading of gel-purified radiolabelled ATP-40-1 RNA onto a 1-ml ATP-C8-agarose column. Plus signs represent complete elution in 3 column-volumes by 0.25 mM ligand; +/- represents partial elution by 3 column-volumes of 1 mM ligand, and minuses indicate no elution by 1 mM ligand over a no-ligand control. Modification of any of six positions on the base or sugar markedly decreases the affinity of the RNA for ATP. Removing the 2' hydroxyl of the ribose is more deleterious than either methylation at this position, or removal of the entire ribose. One possible explanation for this observation is that the 2' hydroxyl acts as a hydrogen-bond acceptor in interacting with RNA. The binding of 2' dATP might then require desolvation of the corresponding donor on the RNA without the compensating formation of a hydrogen bond with the ligand, whereas adenine could still bind weakly as desolvation of the RNA would not be necessary. The 3' hydroxyl is not required for binding, but appears to make a close contact with the RNA as methylation at that position does block binding. In contrast, either removal or methylation of the adenine amino group blocks binding, suggesting that this group may interact with RNA as a hydrogen-bond donor. The triphosphate appears not to interact with the RNA, even though the selection was made on an ATP-agarose resin. But it is likely that there is a high degree of repulsion between the negatively charged triphosphate and the RNA. At the low concentration (5 mM) of Mg²⁺ used in the selection, it may be that the most favourable binding mode involves keeping the triphosphate as far from the RNA backbone as possible.



length and composition, and frequently contained G·U base pairs. The simplest explanation for the observation that all of the selected clones contained a single consensus sequence embedded in a common secondary structure is that these clones contain the shortest sequences capable of binding ATP with the necessary affinity, and that all other sequences with comparable or superior affinity are longer and hence less abundant in the initial random sequence pool.

On the basis of these findings, a smaller RNA of 40 nucleotides (ATP-40-1) was designed, in which the consensus sequence was flanked by stems of six base pairs, with the right-hand stem closed by a stable loop sequence⁴ for enhanced stability (Fig. 2c). This RNA bound ATP as well as the full-length 164-nucleotide RNA C8-ATP-3 and was used for later experiments. Variant 40-oligonucleotide RNAs were also synthesized to test the importance of the highly conserved unpaired G (residue G34 in ATP-40-1) for ATP binding. Deleting this residue or changing it to an A residue eliminated binding, confirming the results of the selection experiments.

To determine which functional groups on the ATP are recognized by the ATP-binding RNA, we examined the ability of a series of ATP analogues to elute bound ATP-40-1 RNA from an ATP-agarose column (Fig. 3). Methylation of positions 1, 2,

3 or 6 on the adenine base, or of the 3' hydroxyl of the ribose sugar, abolish binding, as does removal of the 6-amino or 2' hydroxyl. Positions 7 and 8 on the base can be modified without effect; this is not surprising considering that selection was for binding to ATP linked to an agarose matrix through its C8 position. Adenosine, AMP and ATP are equally efficient at eluting the RNA, suggesting that the 5' position on the ribose moiety is not recognized by the RNA.

We used the methods of isocratic elution³ from ATP-agarose and equilibrium gel filtration⁵ to measure the dissociation constant for the RNA-ATP complex on the column and in solution. The K_d of ATP-40-1 was $\sim 14 \mu\text{M}$ when measured by isocratic elution from an ATP-C8-agarose column (Fig. 4a), and $6\text{--}8 \mu\text{M}$ by equilibrium gel filtration (Fig. 4b). The K_d for the ATP-agarose complex is an upper estimate, because the fraction of bound ATP that is accessible to the RNA is not known. The solution K_d for adenosine was similar to that of ATP ($5\text{--}8 \mu\text{M}$), but the K_d for dATP was not measurable ($>1 \text{ mM}$) (Fig. 4c). The K_d of ATP-40-1 for its ligand dropped to $2 \mu\text{M}$ when the Mg^{2+} concentration was raised from 5 to 20 mM (Fig. 4d). Changing the base pair U18-A33 to C·G, as found in most of the clones initially selected, further decreased the K_d to $0.7 \mu\text{M}$. At almost saturating concentrations of ATP ($50 \mu\text{M}$), the RNA

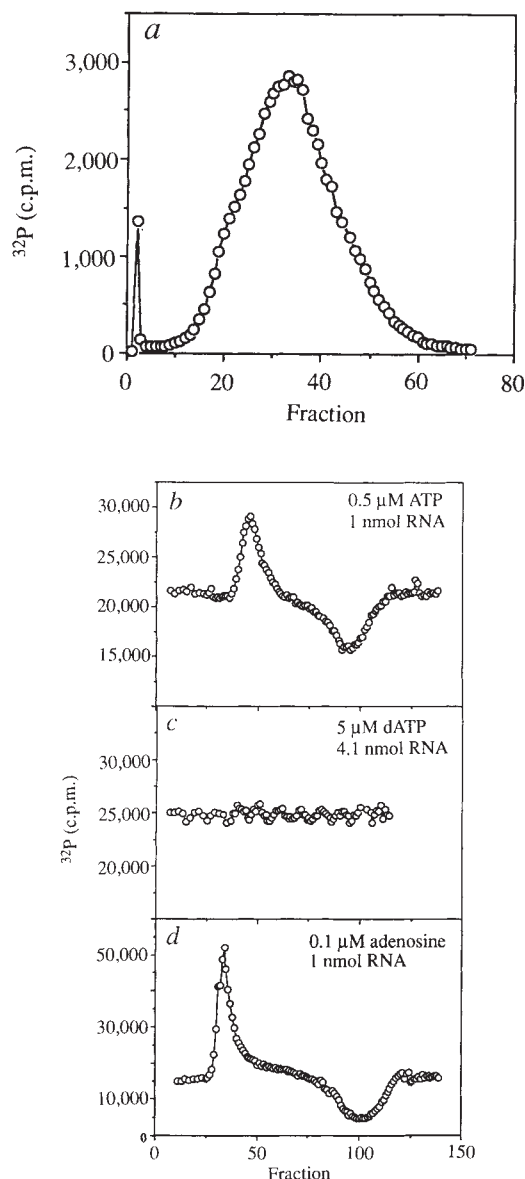


FIG. 4 Dissociation constant measurements. *a*, Isocratic elution. A 2.45-ml ATP-C8-agarose column (diameter 0.5 cm) was equilibrated with buffer (300 mM NaCl, 5 mM MgCl_2 , 20 mM Tris-Cl, pH 7.6). Radio-labelled RNA was loaded on the column and eluted with buffer. 1.95-ml fractions were collected until no radioactivity was detected on the column. The K_d was calculated using the equation $K_d = [L] \times (V_t - V_0) / (V_e - V_0)$ where $[L]$ is the concentration of ligand coupled to the matrix (1.6 mM), V_0 is the void volume of the column (1.9 ml), V_t is the volume of the column, and V_e is the volume of buffer required to elute the RNA. *b*, *c* and *d*, Equilibrium gel filtration. A glass column ($12 \times 0.5 \text{ cm}$; Bio-Rad) containing Sephadex-G25 superfine (Pharmacia) was equilibrated with buffer containing the indicated concentrations of radiolabelled ligand. The indicated amounts of ATP-40-1 RNA were loaded on the column and the radioactivity of individual drops ($28\text{--}30 \mu\text{l}$) was quantified in a scintillation counter. The area under the peak was integrated to calculate the amount of bound ATP and hence the K_d . Buffer contains *b*, $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$; *c*, $[\alpha\text{-}^{32}\text{P}]\text{dATP}$; *d*, 20 mM , instead of 5 mM , MgCl_2 and $2,8,5\text{'-}^3\text{H}$ -labelled adenosine.

bound ~ 0.7 equivalents of ATP; we conclude that the RNA binds its ligand with a stoichiometry of unity.

Kethoxal modification⁶ was used to assess the accessibility of guanosine residues to modification (Fig. 2d). Surprisingly, G7 and G17 within the loop, and G6 (which forms the G·C base pair on the left side of the loop), all of which are strongly protected in the absence of ATP, become highly accessible to modification by this reagent in the presence of ATP. Other guanosine residues, including G8 in the large loop, the single unpaired G opposite the loop, and Gs in the stems, are highly protected in the presence or absence of ATP. These observations suggest that the motif is highly structured both in the presence and absence of ATP, but that binding induces a conformational change in the structure of the RNA.

Much effort has been devoted to the design of abiotic synthetic receptors with specific ligand-binding properties⁷⁻¹⁵. The design of organic receptors for small molecule ligands in non-polar organic solvents has been relatively successful, but it has been more difficult to synthesize receptors that bind in aqueous solution, where hydrogen bonds are critical for specificity but contribute relatively less to binding energy. One of the major problems in small molecule receptor design is the difficulty of providing a framework that is compatible with the precise positioning of numerous functional groups that must interact with the ligand. The secondary and tertiary structures of RNAs and proteins provide a framework for the functional groups that define the ligand binding site. *In vitro* selection, by allowing the sifting of trillions of possible sequences, automatically provides not only a suitable framework but a complete binding-site structure.

As RNA is able to bind ATP with such affinity and specificity, the question arises as to whether this capability is or has been used biologically. We searched for the ATP-binding consensus sequence in structural RNAs listed in the GenBank 73 data base and identified no definitive match. Even if the ATP-binding RNA motif is not used in present day biology, its existence is consistent with theories of a much greater role for RNA in early life forms¹⁶. The frequent appearance of adenosine in enzymatic cofactors has been noted as a possible remnant of the RNA world; as the ATP-binding motif also binds adenosine it could serve to anchor such cofactors to a ribozyme¹⁷. For example, NAD⁺ binds well to the motif, which could therefore serve as one component of a ribozyme with oxidoreductase activity. The ATP-binding motif and other RNA structures capable of binding small molecules in solution could serve as building blocks for the construction of a new set of ribozymes with a wide range of catalytic activities. □

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The EST express gathers speed

Thousands of human genes are being characterized by analysing complementary DNA sequences. But the job of making full use of this wealth of information is just beginning.

THE task of the human genome project — to identify and map all 50,000–100,000 human genes — seems herculean. Substantial headway is being made towards the first of those goals, however, and at an accelerating pace. A good ten per cent of the genes have already been cloned, sequenced (if only partially)¹ and published, due in large measure to the automated complementary DNA sequencing endeavours of several groups, among them Craig Venter's at The Institute for Genomic Research (TIGR). In the July and August issues of *Nature Genetics*, Venter and colleagues have followed up their two earlier reports describing almost 4,000 human brain cDNA sequences^{2,3}, or 'expressed sequence tags' (ESTs), with another 4,500 clones^{4,5}, reflecting an increase in the speed of data gathering and the benefits of subtle changes in sequencing strategy. The August issue also reveals progress in the vital tasks of mapping these sequences⁶ and communicating this wealth of information⁷.

Certain aspects of genome analysis seem suited to private industry, and automated DNA sequencing is one of them. TIGR is barely one year old, but is already generating sequence data at an unprecedented rate. Thirty Applied Biosystems sequencers (which come at \$120,000 apiece) work around the clock to yield up to 1,000,000 nucleotides of sequence per week — about the total submitted to GenBank in the same time. The present output resembles a sort of glorified laundry list — nearly 200 different human cDNA libraries are being analysed and that number may eventually double — but such a compilation is a necessary prerequisite to the vastly more interesting biological analyses that such data will make possible.

Last month, Adams *et al.*⁴ described 3,401 ESTs derived from 3,013 brain cDNA clones, including homologues of the very low density lipoprotein and epidermal growth factor receptors, and two

ESTs bearing significant similarities to the human *KUP* and *Drosophila tram-track* and *Broad-Complex* genes, which encode zinc-finger proteins. Another newly discovered zinc-finger gene, *LAZ-3*, bearing homology to these *Drosophila* transcription factors, is disrupted in lymphoma translocations involving chromosome 3 (ref. 8). Adams *et al.* also offer a glimpse of things to come with a 'computerized northern blot' — a comparison of the sequence profile from various tissues, such as brain versus liver. Structural and regulatory proteins are more abundant in brain, but secreted proteins and polypeptides involved in protein synthesis predominate in liver.

In this month's issue, Adams *et al.*⁵ describe how they found almost the same number of homologies while analysing only half as many clones. The trick is to use a directionally cloned brain cDNA library, which optimizes the amount of coding information obtained from an EST (and hence the likelihood of detecting a significant homology) and also minimizes the chances of contamination or of generating chimaeras. Adams *et al.* identified clones of a second amyloid precursor-like protein (APLP), a cousin of the amyloid precursor protein so central to Alzheimer's disease pathology. The complete characterization of this APLP2 cDNA isolated by more traditional means will soon be reported⁹.

The impact of this strategy is considerable: including a wealth of unpublished data, Venter estimates that some 25,000 human gene sequences have been identified, the vast majority ESTs. As recent findings from large-scale genomic sequencing projects point to there being a likely total of about 75,000 human genes¹⁰, perhaps as many as one-third of human genes have already been isolated. But lest researchers be discouraged that the more laborious pursuit of single genes is now redundant, they may take heart from the fact that Venter's group has yet to spot the recently discovered Huntington's gene among their brain cDNAs.

But as these EST compilations grow, the geneticist may ask what use they serve if there is no cohesive attempt to map them onto the genome. Some groups are rising to the challenge. Polymeropoulos *et al.*⁶ have mapped more than 300 brain ESTs to whole chromosomes using the polymerase chain

reaction on somatic cell hybrids. Their distribution, however, does not fully correspond with the cytogenetic length of the chromosomes — for example, chromosomes 2 and 19 contain similar numbers of genes despite their large difference in size. And chromosomes 13 and 18 were relatively under-represented in mapped genes, suggesting that the only viable human trisomies are those of chromosomes 13, 18 and 21, due to their paucity of genes. Some researchers are beginning to localize ESTs to sub-chromosomal regions, especially on the X chromosome. But even though this huge resource begs to be exploited, there is still no systematic undertaking to map ESTs. Even if it takes several years to compile the precise localization of thousands of ESTs, such knowledge would change the face of medical genetics: mapping rare disease traits, rather than isolating the corresponding genes, would become the rate determining factor.

Aside from accelerating the pace of gene discovery, the EST bandwagon holds great promise for work with other organisms¹ (including *Caenorhabditis elegans* and *Saccharomyces cerevisiae*) and in other disciplines (such as the study of changes in gene expression associated with cell division and tumorigenesis). The need for direct access to EST data will hasten the day when biologists embrace computer databases as a regular part of their research. Such a database, called dbEST, is now available⁷; as well as being a repository for EST data, dbEST periodically conducts homology searches against new entries in nucleotide and protein databases. That still leaves the unenviable task of deciding how long to devote sizeable portions of journals (not the CD-ROM variety) to expanding lists of ESTs. But then the life of an editor is never easy.

Kevin Davies

Kevin Davies is Editor of Nature Genetics.

Also in this month's *Nature Genetics*: three reports on the correlation between repeat size and age of onset in Huntington's disease; a single gene for Hirschsprung disease located on chromosome 10; the first missense mutation identified in dystrophin; verifying the accuracy of chromosome physical maps; and mapping the ovine fecundity gene.

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Multiplexed biochemical assays with biological chips

Stephen P. A. Fodor, Richard P. Rava, Xiaohua C. Huang, Ann C. Pease, Christopher P. Holmes and Cynthia L. Adams

High density peptide and oligonucleotide chips are fabricated using semiconductor-based technologies. These chips have a variety of biological applications.

CONVENTIONAL high-throughput assay systems (for example, immunoassays, enzyme assays, DNA reverse dot blots) usually involve highly repetitive binding measurements in a microtitre format. This requires preparation of large quantities of reagents, followed by deposition into individual microtitre wells. Because of the manual effort required to prepare these assays, the number of tests is usually limited, and customarily performed in the 96-well format. Both academic and commercial laboratories have put substantial effort into automating, miniaturizing and expanding the multiple assay format.

Recently, we have developed new technologies to synthesize and assay biological molecules in a miniaturized format¹. The method uses light to direct the combinatorial chemical synthesis of biopolymers on a solid support. The identity and location of each biopolymer is known, and its interaction with a molecular binding agent can be measured. These miniature biological arrays, or chips, can then be used for a variety of multiplexed biochemical assays, including epitope mapping, the development of chemical analogues, genetic diagnostics and sequencing by hybridization.

In order to fully implement this technology, two key methodologies were developed. The first, light-directed combinatorial

chemical synthesis, enables the synthesis of tens to hundreds of thousands of compounds in precise locations on the chip. The second technology, laser confocal fluorescence scanning, facilitates the measurement of the molecular binding events at individual sites on the array. The combination of these two technologies forms the core of new instrumentation for a miniaturized, multiplexed assay format that can be utilized for research, diagnostics and bioanalytical studies.

Light-directed synthesis

Light-directed chemical synthesis employs two mature technologies: semiconductor-based photolithography and solid-phase chemical synthesis. Synthesis linkers modified with photochemically removable protecting groups are attached to a solid substrate (Fig. 1). Light is directed through a photolithographic mask to specific areas of the synthesis surface effecting localized photodeprotection. The first of a series of chemical building blocks (for example, photoprotected amino acids or hydroxyl photoprotected deoxynucleosides) is incubated with the surface, and chemical coupling occurs at those sites which have been illuminated in the preceding step. Next, light is directed to a different region of the substrate through a new mask, and the chemical cycle is repeated. Highly efficient synthesis strategies can be employed that will generate a maximum number of compounds in a minimum number of chemical steps. For example, the complete set of 4ⁿ polynucleotides (length n), or any subset of this set can be produced in only 4 × n chemical steps. The patterns of illumination and the order of chemical reactants ultimately define the products and their locations.

The precision and high resolution of photolithography allows miniaturization of the synthesis scale, and thus, thousands of biopolymers reside in highly compact matrices. A target molecule can now be incubated with the array, allowing the assay to be carried out against all members simultaneously. The assay molecule can be labelled with a fluorescent reporter group to facilitate the identification of specific interactions with individual members of the matrix. This technology requires only minute preparation and consumption of chemical reagents as well

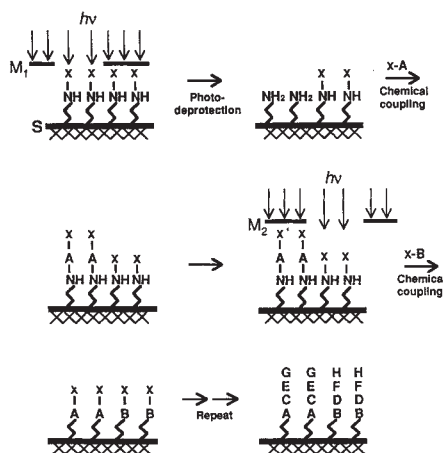


FIG. 1 Light-directed chemical synthesis scheme. Light is used to direct the synthesis of a large number of peptides or oligonucleotides in precise locations in a miniaturized and accessible format.

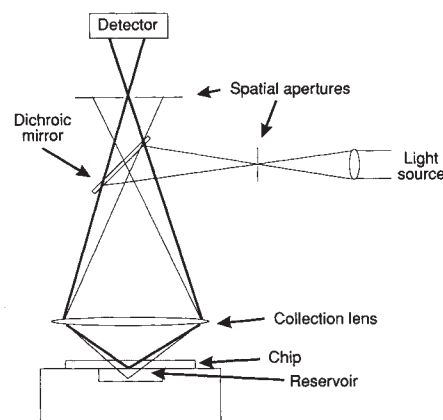


FIG. 2 Laser confocal fluorescence detection scheme. After synthesis of the array, the chip is mounted on a thermostatically regulated flow cell. Fluorescently labelled target molecules (for example, antibodies, receptors, single-stranded DNA) are introduced into the reservoir, and the surface of the chip is interrogated for binding (fluorescence signal) as described in the text.

as minute preparation and consumption of biological targets.

Confocal fluorescence detection

The interaction of biological targets to compounds on the chip is best characterized in terms of kinetics and thermodynamics. This necessitates interrogation of the array while in direct contact with a solution of target molecules. The detection system must be extremely sensitive, and capable of discriminating between surface-bound molecules and those free in solution. Laser confocal fluorescence microscopy meets these criteria.

In the assay scheme, the array is inverted on a temperature-regulated flow cell against a reservoir containing the fluorescently tagged target molecules (Fig. 2). Laser excitation enters through the back of the array, focused at the interface of the array surface and the target solution. Fluorescence emission is collected by a microscope objective, and passes through a series of optical filters to a sensitive detector, such as a photomultiplier tube. Out-of-focus fluorescence emissions (for example, from the target molecule solution) are not efficiently transmitted through the confocal aperture to the detector. By simply scanning the laser, or translating the flow cell, a two-dimensional

fluorescence image of target binding is generated in a matter of minutes.

Applications

There are many potential applications of high-density chemical arrays. Two types of arrays are currently being produced: peptides¹ and oligonucleotides^{1,2}. In the following examples, the uses of peptide chips for epitope mapping and oligonucleotide chips for DNA sequence analysis are demonstrated.

Epitope mapping. Understanding the structure-activity relationship (SAR) between a ligand and its biological macromolecular partner (antibody, receptor, etcetera) can be a time-consuming task. A large number of synthesized variants of a ligand are required to map completely the important recognition sites of the epitope. Peptide chips allow the rapid generation of a large SAR database for mapping the epitope of biological macromolecules.

As an example, Fig. 3 presents a fluorescence image generated from the monovalent binding of fluorescently labelled Fab fragments (from antibody D32.39) to an array of

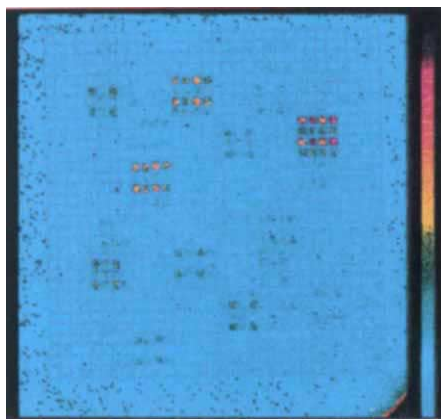


FIG. 3 Peptide chips. Fluorescence image of an antibody Fab bound to an array of 1,024 peptides. The specificity and intensity of binding defines the antibody epitope.

peptides. This antibody is known to bind the opioid peptide, dynorphin B. In this experiment, ten chemical synthesis cycles yield a chip with all 1,024 linear sub-sequences contained in the sequence FLRRQFKVVT³. All 1,024 sequences are in a square measuring 1.28 cm × 1.28 cm. The most intense features on the array incorporate the sequence RQFKVVT, consistent with solution mapping studies on a limited number of peptides³. The incubation of Fab with the array, detection of binding, and identification of both the correct antibody epitope and additional high-affinity analogues were carried out in less than three hours. Less than 500 ng of Fab is required to carry out all 1,024 assays.

Sequencing by hybridization. An exciting application of oligonucleotide arrays is in DNA sequencing. Conventional DNA sequencing methodology is labour intensive, and requires electrophoretic size separation of labelled DNA fragments. An alter-

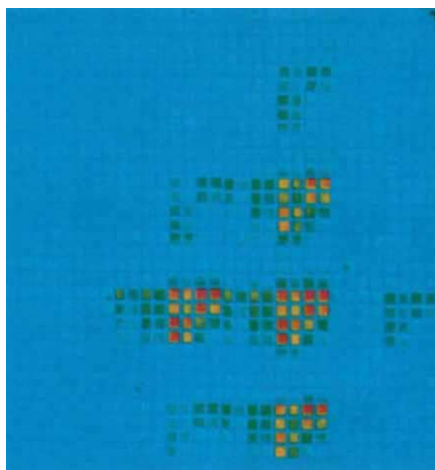


FIG. 4 DNA chips. One picomole of the target molecule 3'-AACCCAAACCC-fluorescein was incubated with an array of octanucleotides. High fluorescence intensity indicates binding to complementary probe sequences.

native approach, termed 'sequencing by hybridization' (SBH), employs a chip containing a set of short oligonucleotide probes to generate hybridization patterns from the complementary sequences contained within a longer target strand of DNA⁴⁻⁸. The hybridization pattern is then used to reassemble the original target DNA sequence.

To test the feasibility of this approach, a chip was fabricated displaying all 8-mer combinations of the nucleosides, guanine and cytosine⁹. After synthesis, the chip was incubated with 1 pmol of 3'-AACCCAAACCC-fluorescein, and scanned at 15 °C. Hybridization of this target to the four complementary 8-mer sub-sequences, and many of their single base mismatches, is observed as brightly fluorescent spots on the image in Fig. 4. Methods and algorithms to reconstruct the target sequence from such hybridization data are currently being developed⁹.

Conclusions and prospects

Process methods for the fabrication of miniaturized peptide and oligonucleotide arrays have been developed. The current photolithographic process generates array densities in the range of 10⁵ synthesis sites per cm². There is no fundamental reason why the size of the synthesis sites cannot approach the size features regularly achieved in semiconductor fabrication. A moderate increase in our present resolution capabilities will easily allow 10⁶ assays per cm² to be carried out. Furthermore, the implementation of photolithography with solid-phase chemical synthesis, lends itself to the parallel manufacturing practices of the semiconductor industry.

The current explosion in understanding the molecular basis of disease is spawning exciting new opportunities for the use of oligonucleotide chips in DNA sequence analysis. These include DNA mapping, DNA mutational analysis, sequencing, sequence

checking and medical diagnostics. The versatility in the construction of oligonucleotide arrays, allows one to design chips to read specific genes. For example, a ten-kilobase gene consists of an overlapping set of 10,000 oligonucleotide probes, well within the current chip fabrication technology. As the structure of the genome is elucidated, the chip technology will allow enormous quantities of genetic information to be stored on and read from the surface of a single chip. □

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and M13 helper phage allows recovery of the pBK-CMV phagemid vector containing the cloned genetic insert. Prokaryotic expression is driven by the IPTG-inducible *lacZ* promoter; eukaryotic expression by the cytomegalovirus immediate early promoter. Stratagene says that the Zap Express lambda vector facilitates the construction, amplification and screening of libraries. Unique *Eco*R I, *Xho* I and *Spe* I sites allow unidirectional cloning for sense and antisense cDNA libraries. The *Bam*H I site allows cloning of *Sau*3A-digested genomic DNA, and the *Sma* I site allows cloning of blunt-end polymerase chain reaction fragments. Both the Zap Express and pBK-CMV vectors contain T3/T7 promoters for *in vitro* transcription and an *f1* origin of replication for purification of single-stranded DNA. Other features include blue/white colour selection of clones with insert, LacZ fusion protein expression, neomycin resistance for prokaryotic selection and G418 resistance for eukaryotic selection.

■ Probes

Texas Fluorescence Laboratories, a new company that specializes in **fluorescent ion indicators, ionophores, probes for cell signalling** and general custom synthesis, has a new catalogue for 1993–1994 (*Reader Service No. 107*). FIM-1 and RIM-1 are new fluorescent probes for protein kinase C (PKC), which exhibit good specificity and provide a rapid and simple means of visualizing the distribution of PKC in the cell. The characterization and use of these dyes was published in the 25 July issue of the *J. biol. Chem.* FFP-18 is a new amphipathic Fura-2 analogue with a hydrophobic tail, which, TEFLABS says, appears to function as a near-membrane calcium indicator upon injection into cells. FF6-AM loads and acts like Fura-2-AM but is a little more hydrophobic. It is not, however, as hydrophobic as FFP-18. PE-3-AM is a new fura analogue that resists leakage and compartmentation. Cells that extrude Fura-2-AM over a time-course of 30 min remain loaded with PE-3-AM for hours, according to the company. SQI-PR and SQI-ET are two new synthetic ionophores for sodium, whose responses compare favorably with that of valinomycin for potassium. TEFLABS is also providing TFL-Zn, a new water-soluble version of the histochemical stain TSQ. In addition to these new probes, indicators for Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , and pH, and Ca^{2+} ionophores and NMR probe are available. TEFLABS also performs custom synthesis of organic compounds.

Applied Imaging and Cytocell announced earlier this year that, under a new strategic agreement, Applied Imaging will distribute Cytocell's **DNA probe kits** (*Reader Service No. 108*). These Chromoprobe kits are based on a method of reversibly binding DNA probes on a special-purpose coverslip; this makes for a convenient method of applica-

tion to sample slides. With the kits, denaturation and hybridization occur in one stage, without the need for time-consuming probe preparation and pipetting. The probes are directly labelled with fluorescein and require no additional labelling or amplification steps. A further benefit of the kits is the rapid hybridization time of 1 hour and an application time of 1.5 hours. Kits are available now for the following chromosomes: 1, 8, 12, 18, 13/21, X, Y with a specific 21 probe and a range of chromosome paints.

■ Image makers

A new **fluorescence-based imaging instrument**, the FluorImager 575, is now available from Molecular Dynamics (*Reader Service No. 109*). The instrument reads fluorescence-labelled DNA and protein



FluorImager 575 directly scans samples up to 20×25 cm in less than 5 min.

molecules directly from acrylamide and agarose gels, thin layer chromatography and microtitre plates, and membranes. As the instrument scans samples (of up to 20×25 cm) directly, the often time-consuming exposure and processing steps involved in autoradiography are eliminated. Also, the two-channel detection capability of the instrument allows researchers to double the amount of information obtained from one experiment. Alternatively, the two-channel feature can be used to improve accuracy by running standards and samples together in the same lane or microtitre well. Molecular Dynamics says that the signal response of the FluorImager 575 is quantitative over four orders of magnitude. Specially developed FluorKit reagents, which have been developed by Promega, are available from Molecular Dynamics. The first four cover broad-based DNA mapping applications (DNA fragment analysis on electrophoretic separations and DNA quantitation analyses on 96-well microtitre plates) and two enzyme activity assays (the chloramphenicol acetyltransferase reporter gene assay for thin layer chromatograms and the protein kinase assay for agarose gels). ImageQuANT analysis software — designed to simplify quantitation and facilitate the transfer of data to Windows and Macintosh programs — completes the system.

Along the same lines is the FMBIO FluorImager from Hitachi Software Engineering America — a filmless, **nonradio-**

active image analysis system that is designed for northern, Southern and western blots, one- and two-dimensional gels, thin layer chromatography plates, chloramphenicol acetyltransferase (CAT) assays and DNA sequencing (*Reader Service No. 110*). Hitachi says that FMBIO takes only 30–90 min to generate images, compared to autoradiography which can require film exposures of one to two days. The system consists of a fluorescent imaging unit, a Sun SPARCstation computer, PDI Discovery Series image analysis software and a complete line of FMBIO FluorImager reagent kits developed by Molecular Probes. Other features of the FMBIO analyser are a 20×47 cm reading area, a laser life of 10,000 hours, excitation and emission wavelengths of 532 nm and 605 nm, respectively, and a dynamic range of four orders of magnitude. Software programs are offered for one-dimensional blot and gel analysis, restriction fragment length polymorphism and DNA fingerprint analysis, DNA sequence reading analysis, and two-dimensional gel comparisons and databasing. FMBIO reagent kits have been developed for CAT assays, western blots, DNA staining, oligonucleotide labelling and DNA sequencing.

Bio-Rad's Model GS-670 **imaging densitometer** offers rapid graphical imaging and quantitation capabilities for a wide range of sample types and sizes, including one- and two-dimensional gels, autoradiographs, wet gels, dot or slot blots, thin layer chromatography plates and western blots (*Reader*



Bio-Rad's personal densitometry system.

Service No. 111). The densitometer features variable resolution scanning, which allows the user to control the computer file size and the scanning speed (1–15 min, depending on the selected resolution, and possible $29.5 \text{ cm} \times 42 \text{ cm}$ scanning area). Bio-Rad says that coloured gels and blots do not pose problems for the variable wavelength light source. The featured wavelength range from 400 nm to 750 nm is designed to eliminate the difficulties in visualizing and quantifying a variety of stains. The system is equipped with Molecular Analyst/PC or Macintosh image analysis software, which is designed to provide quick image optimization, accurate volume or area integration for single bands or whole lanes of data, complete molecular weight, concentration and pI determination,

data export and the printing of publication-quality reports.

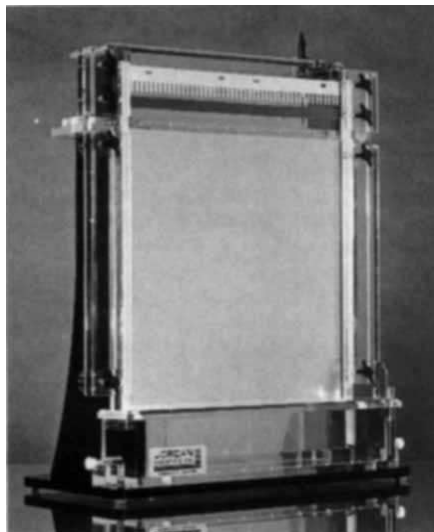
Canberra Packard's new InstantImager **autoradiography system** electronically locates and measures radiolabelled macromolecules in gels, blots, membranes, thin layer chromatography plates, tissue slices and other two-dimensional samples (*Reader Service No. 112*). This new imaging technology eliminates the use of X-ray films and storage phosphor screens from autoradiography work. Sample sizes up to 20 × 24 cm are directly loaded in the system. A microchannel array detector, invented by Professor A. Jeavons of Oxford, UK, images and quantitates the distribution of radioactivity; the formation of autoradiography images can be followed continuously on a high-resolution CRT display. The company says that the InstantImager is 100 times faster than exposure to X-ray film and ten times faster than phosphor screen imaging. Activities as low as 0.02 c.p.m per mm² can be analysed in less than an hour, and spots or bands, which differ in intensity by a factor of 100,000 can be analysed simultaneously. The detection system is interfaced to a 486DX/66 MHz PC. Images are displayed and printed in continuous tone black and white, or 256 colours. InstantQuant, a Microsoft Windows-based imaging software package, completes the package. Results can be transferred to other programs such as Excel spreadsheets, and images can be converted to TIF formats for desktop publishing with a Macintosh or PC.

■ Sequencing

A new **DNA sequencing kit**, the Ladderman dideoxy sequencing kit, which uses a novel DNA polymerase, *Bca* DNA polymerase, has been developed by Takara Shuzo and is being distributed by British Bio-technology Products (*Reader Service No. 113*). The company says that *Bca* DNA polymerase operates at high optimum temperatures and is highly processive so that longer, clearer sequence ladders can be produced without nonspecific steps caused by template secondary structure. In addition, the enzyme

has a rapid reaction rate and the high efficiency of primer use allows uniform ladders to be produced from <0.2 pmol of DNA template per reaction. No annealing step is required and extension and labelling can be performed simultaneously.

The PhorRunner **sequencing gel system** from Jordan Scientific features built-in aluminium thermoplates that are designed to distribute heat evenly, so that even banding patterns are obtained across the entire width of the gel (*Reader Service No. 114*). Each sequencer can be adjusted to run gels 42 cm

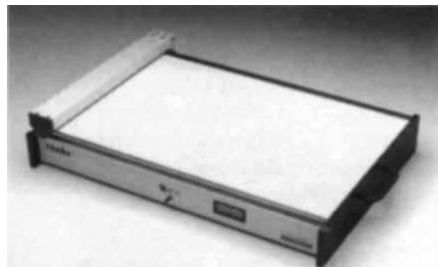


PhorRunner sequencing gel system comes with built-in thermoplates.

or 62 cm in height — a design feature that should provide added flexibility. Other features include a built-in clamping system, easy-to-drain removable buffer chambers, interlocking safety covers and a heavy-duty construction. The Sub-Mini IV is a gel system that can run one or two double-width minigels. Up to 54 samples can be run in as little as 30 min, which makes this system suitable for procedures in which large numbers of samples need to be run quickly, such as PCR analyses. The system is equipped with Jordan Scientific's QuickCast System,

which not only provides tapeless gel casting but also includes a built-in levelling system to ensure that gels are cast evenly. A wide selection of interchangeable combs and gel beds are available.

Hoefer Scientific's TE 90 GeneSweep **sequencing gel transfer unit** transfers DNA electrophoretically from polyacrylamide sequencing gels to nylon membranes (*Reader Service No. 115*). DNA on the membrane can then be probed for standard or multiplex sequencing, or for genetic mapping and typing. Probes can be detected with



Hoefer Scientific's GeneSweep sequencing gel transfer unit.

chemiluminescent, colorimetric or conventional radioisotopic methods. With GeneSweep, an arm sweeps the spring-loaded positive electrode across a semi-dry gel membrane stack that rests on the negative electrode platform. The arm adjusts to the thickness of the stack so that DNA transfers evenly and completely, even from wedge gels. The unit comes with a built-in power supply that delivers up to 2 A. Hoefer Scientific says that transfer from a 34 × 50 cm gel takes only 10 min. The company also supplies blotting paper and nylon membranes, sized for standard DNA sequencing gels.

■ Miscellaneous reagents

Ampligase **thermostable DNA ligase** from Epicentre Technologies permits the detection of trinucleotide repeat sequences associated with several human genetic diseases such as Huntington's disease, fragile X syndrome, myotonic dystrophy, spinobulbar muscular atrophy and spinocerebellar ataxia

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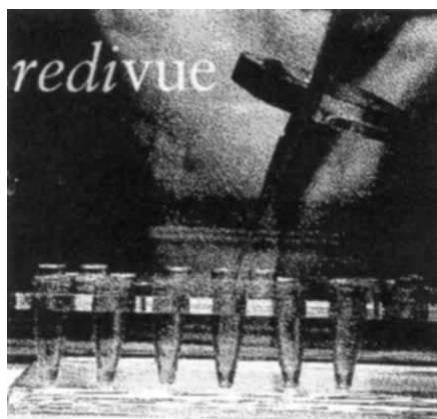
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PRODUCT REVIEW

type 1 (*Reader Service No. 116*). Epicentre says that by using Ampligase with the new technique of repeat expansion detection, researchers have been able to identify and size triplet repeats directly from genomic DNA without prior knowledge of their chromosomal location. The enzyme is highly specific because it lacks activity on blunt-ended DNA. It is also sensitive because its thermostable properties permit amplification during extended thermocycling. The company says that activity is retained even after 500 cycles or 16 hours of cycling. These two characteristics — specificity and thermostability — make it suitable for exponential amplification and detection of defined DNA sequences by ligation amplification or ligation chain reaction.

Amersham's Redivue coloured ^{32}P -nucleotides contain an intense, inert red dye and stabilizer formulation that is designed to



Amersham is seeing red with its Redivue coloured ^{32}P nucleotides.

improve significantly visualization of the nucleotide solution during use (*Reader Service No. 117*). Inclusion of the red dye means that nucleotide is clearly visible in reaction mixes, even at concentrations as low as 1 μl in 50 μl , according to Amersham. The addition of a stabilizer allows the nucleotides to be stored in a convenient liquid form at +4 °C; this eliminates the need to thaw nucleotides before use. Amersham states that as a result of extensive testing, Redivue nucleotides have been shown to perform at least as well as standard nucleotides in a wide range of applications.

Vuman is the new facility established within the biochemistry and molecular biology department at the University of Manchester that is offering the scientific community services in **oligonucleotide synthesis, peptide synthesis and protein sequencing** (*Reader Service No. 118*). The oligonucleotide service includes synthesis, deprotection and recovery with guaranteed yields of 20 nmol. The synthesized oligos are suitable for the polymerase chain reaction, sequencing and mutagenesis without the need for further purification. Synthesis, cleavage from resin and recovery by precipitation with ether followed by lyophilization are part of the peptide synthesis service. A minimum purity of 70 per cent is guaranteed

by the facility. Mass spectroscopy is used to confirm complete synthesis and accurate molecular weight of peptide. Future plans include the use of capillary electrophoresis profiles. Completing the line-up of services is the protein sequencing service. The services provided include sequencing of purified proteins or blotted protein bands and data analysis.

The pharmaceuticals and fine chemicals division of Genzyme last month announced details of its new line of **OneSource phospholipids** — a complete range of synthetic and natural phospholipid products with extensive regulatory, analytical and manufacturing support for pharmaceutical applications (*Reader Service No. 119*). The synthetic phospholipids were developed by Genzyme utilizing funding from Neozyme. This technology was reacquired by Genzyme in December last year. The natural phospholipids being offered are provided as the result of a strategic agreement between Genzyme and the Swedish company, Karlshamns LipidTeknik. In addition to the range of phospholipid products, Genzyme has developed technology for the derivatization of phospholipids with other molecules of pharmaceutical importance.

Progenius is the new **DNA purification kit** from Bio/Gene (*Reader Service No. 120*). The method used in the kit is based on a procedure described by Vogelstein and Gillespie (*Proc. natn. Acad. Sci. U.S.A.* **76**,



"Progenius" DNA purification kit.

615;1979). Bio/Gene says that the silica-based DNA purification matrix has a loading capacity in excess of 3 μg DNA per mg. The silica has been size-fractionated and purified to meet quality assurance standards. Stated uses of the kit include the isolation of DNA fragments from agarose gels, the removal of unincorporated radiolabelled nucleotides, the preparation of genomic DNA samples for gene amplification procedures and for rapid mini and midi preparations. The procedure is designed to take less than one hour from start to finish. ☐

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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